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THE JOURNAL OF APPLIED BACTERIOLOGY

(Formerly Proceedings of the Society for Applied Bacteriology)

EDITED FOR THE SOCIETY FOR
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These concern the paper by Norah Thompson, entitled "The Influence of Acidity on the Multiplication of Lactic Streptococcal Bacteriophage in Liquid Media", in Vol. 22, no. 2, pp. 287-293.

On p. 287, line 10; for 'adsorption at pH 7' read 'adsorption at pH 5.'

On p. 292, line 3; for '35' read '25'.

On p. 292, line 5; for '40' read '30'.

On p. 292, line 26; for 'dells' read 'celtis'.

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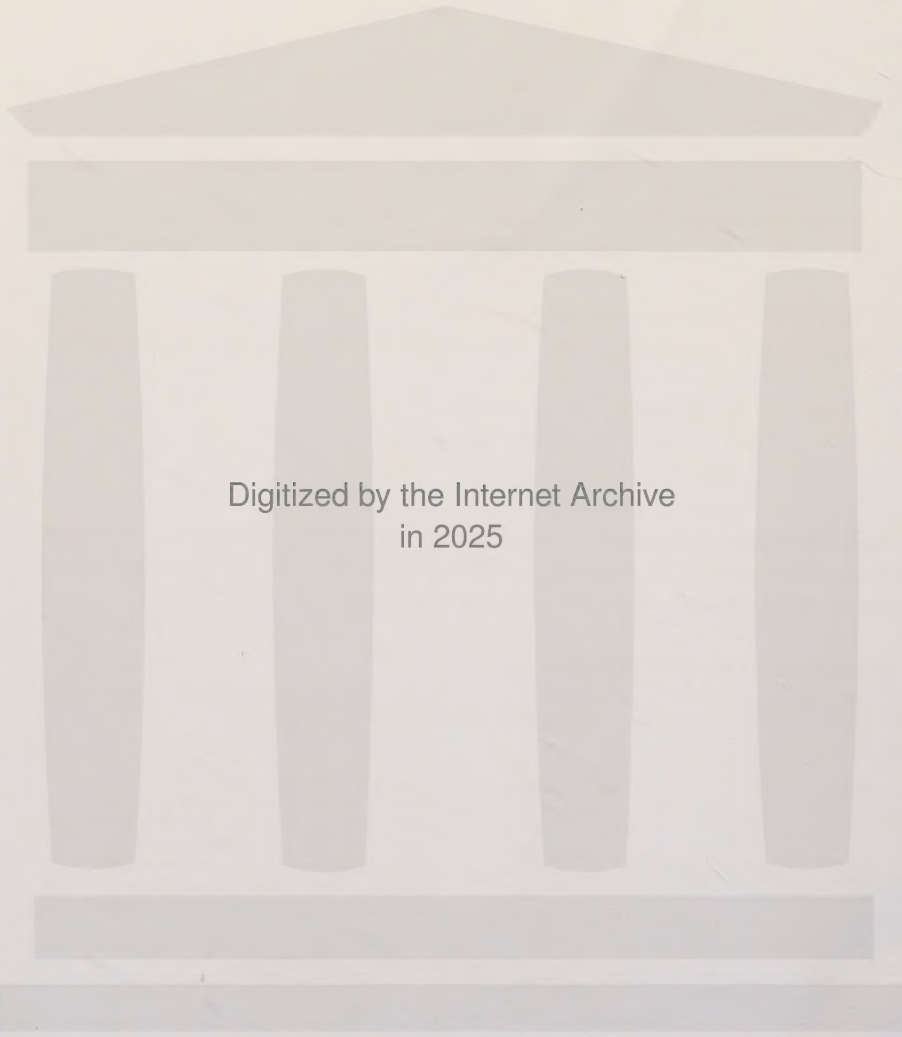
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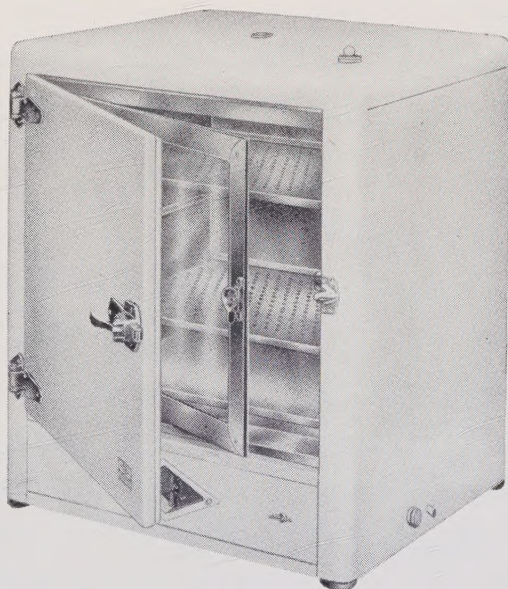
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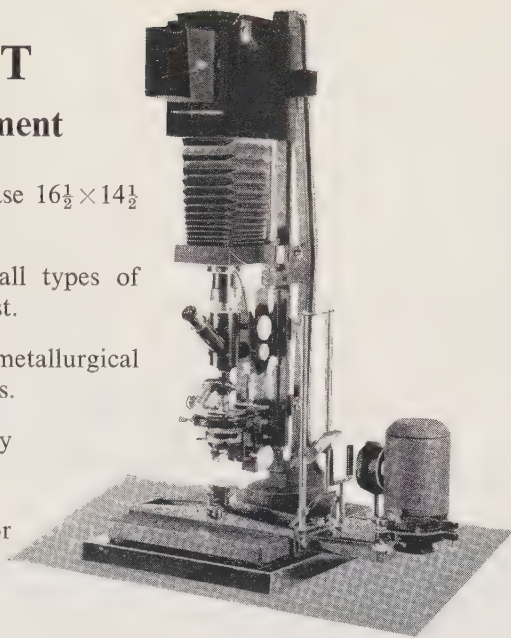
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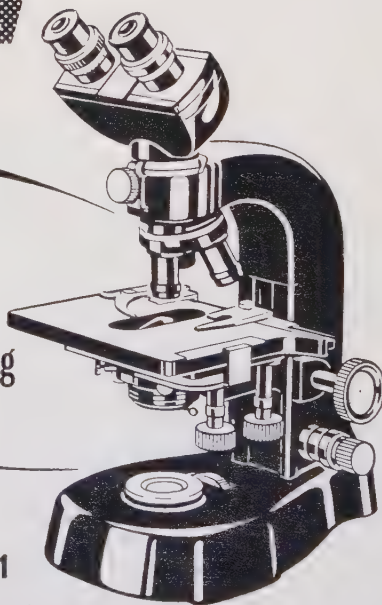
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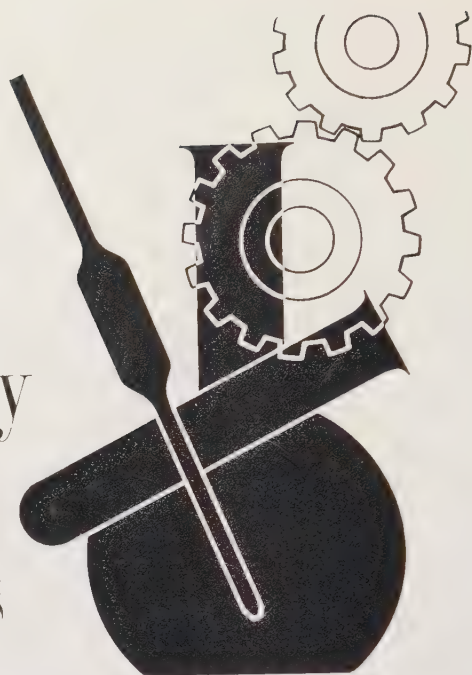
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(Symposium on The Lactobacilli: Paper I)

AN APPROACH TO THE CLASSIFICATION OF THE LACTOBACILLI

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1. INTRODUCTION

THE VALUE of exact identification of species is twofold—first, in defining a common universe of discourse, and second, because an understanding of their ecological and physiological relationships is implicit in the identification of species and thus often leads to a clearer statement of fundamental problems in bacterial metabolism. The purpose of any concept of a genus should be to recognize and relate it objectively and naturally to other taxa of micro-organisms through an understanding of modal characteristics. This statement implies that a concept of a taxon is both a biological and a statistical one. Therefore, by correlating a sufficient number of typical characteristics a certain small degree of variability falls into its proper perspective, since adequate information enables us, despite minor variability, to predict modal behaviour with high accuracy.

2. DEFINITION OF THE GENUS

Our definition of the genus *Lactobacillus* will be modified from that found in Bergey's Manual (Breed, Murray & Hitchens, 1948; Breed, Murray & Smith, 1957). The genus *Lactobacillus* is a member of the family Lactobacteriaceae (Orla-Jensen, 1921) and the tribe Lactobacilleae (Winslow *et al.* 1920). It has been previously divided into three subgenera, namely *Thermobacterium* (Orla-Jensen, 1919), the members of which are homofermentative, with relatively high optimum and minimum growth temperatures and maximum growth temperatures generally in the range 45–50°; *Streptobacterium* (Orla-Jensen, 1919), whose members are also homofermentative but with generally lower maximum and minimum growth temperatures and a wider variety of fermentation reactions; and *Betabacterium* (Orla-Jensen, 1919), with heterofermentative species, producing only 50–65% of DL-lactic acid and relatively large concentrations of other products from the fermentation of carbohydrates, namely acetic acid, CO₂, and certain minor products such as ethanol.

The lactobacilli are nonsporulating aciduric rods of varying length, which divide in one plane only, without branching, clubbing or bifid formation. They are Gram-positive, becoming Gram-negative with increasing age and acidity. Short rods often appear as coccobacilli and chain formation is common, particularly in the later logarithmic phase of growth: thus, the name *Streptobacterium* seems unfortunate for one of the taxa previously considered a subgenus (Breed, Murray & Hitchens, 1948). Growth is favoured by anaerobic or micro-aerophilic conditions or an atmosphere of CO₂. Complex nutritional requirements for amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acids or fatty acid esters, and fermentable carbohydrates, are characteristic. Glucose is fermented, indole is not produced, gelatin is not liquefied and nitrates are not reduced, except for certain strains of *L. plantarum* in special media (Costilow & Humphreys, 1955). They are catalase negative except that a feeble decomposition of peroxide by three New Zealand strains was observed by Dacre & Sharpe (1956); however, the characteristic haem spectrum has not been proved, and this is indispensable for characterizing an enzyme as catalase. No significant fermentation of lactate occurs: this would exclude such organisms as *L. bif fermentans* (Pette & van Beynum, 1943), which has both vigorous carbohydrate and lactate fermentations, and such an organism would therefore be more properly allocated to another genus. Pigment production is rare, but when it is present the colour is yellow or orange to rust or red. Motile varieties with peritrichous flagella have been reported occasionally (Harrison & Hansen, 1950; Mann & Oxford, 1954), but on detailed examination these have been otherwise atypical in one or more respects. The habitat may be dairy products and effluents, grain and meat products, water, sewage, beer, wine, fruits and fruit juices, fermenting vegetables, the alimentary tract and vagina of man, bovines and rodents, and the intestinal contents of fowls and pigs.

3. COMMENTS ON METHODS FOR STUDYING LACTOBACILLI

General methods for the study of the lactobacilli have been well described by Orla-Jensen (1919, 1943) and by Orla-Jensen, Otte & Snog-Kjaer (1936), and more recently by Rogosa *et al.* (1953), Sharpe (1955a), and the group of investigators at the National

Institute for Research in Dairying (Wheater, 1955*a,b*; Briggs, 1953). These methods will not be described in detail here, but certain points deserve some attention and emphasis.

The primary isolation of lactobacilli is now possible on highly selective media (Rogosa, Mitchell & Wiseman, 1951*a,b*; Keddie, 1951; Mabbitt & Zielinska, 1956). However, these media contain considerable quantities of acetate and aerobically incubated streak plates are nearly always unsuccessful since evaporation so concentrates the acetate at the surface of the medium that even lactobacilli are inhibited. Incubation in an atmosphere of 95% H_2 +5% CO_2 or 95% N_2 +5% CO_2 serves to prevent surface evaporation of plates and also to provide favourable anaerobic or micro-aerophilic conditions and a stimulating CO_2 tension. In the study of fermentation reactions Orla-Jensen (1919) had already clearly demonstrated the necessity for an adequate nutritional environment. The importance of an adequate, balanced basal medium and of bringing cultures to a vigorously growing state by repeated transfers of large inocula can hardly be overstressed. For instance, poor and variable growth of *L. viridescens* is inevitable except when the proper concentrations of citrate, manganese and thiamine are available to the organism (Niven & Evans, 1957). In addition to this, however, the pH of the basal medium can determine the enzymic activity on available substrates. We have found that an initial pH of 6.4 and the use of chlorophenol red (0.004%) give satisfactory conditions for general purposes. But there is no doubt that a relatively low pH (5.5–6.0) is most favourable for carbohydrate fermentation in sufficiently buffered media. This is true also for growth (Orla-Jensen, 1943; Rogosa *et al.* 1953). It should not be forgotten that the lactobacilli are aciduric. Thus, a simple and common way to induce variation in certain fermentation reactions, particularly but not exclusively with the heterofermentative lactobacilli, is to use neutral media. Unfortunately, an altogether reliable indicator, free of major salt and protein errors, is not available for the pH range 5.5–6.0 and it may be necessary to determine the final pH electrometrically. However, this may have its advantages since a quantitative assessment of acidity indicates the vigour of a fermentation and this is often characteristic of a species. For example, the fermentation of arabinose by *L. brevis* results in a final pH at least 0.5 units lower than is the case for glucose (Rogosa *et al.* 1953).

Present basal media for fermentation reactions contain acetate and oleic acid esters, specifically Tween 80, and have a pH value which inhibits the growth of spore-forming bacilli, so that they require only minimal heat treatment, such as momentary autoclaving. In these circumstances most carbohydrates may be added directly to the medium before heating: but arabinose, fructose, galactose, maltose, mannose, rhamnose, sorbose and xylose must not even be brought into solution by heat and must be sterilized by filtration to achieve reliable results. Also, the concentration of certain carbohydrates, galactose and lactose for example, should be high enough (1.4–2.0%) to permit the adaptive fermentations which sometimes occur only in the presence of sufficient substrate (Rogosa *et al.* 1953).

In the test for NH_3 production from arginine, so characteristic of most heterofermentative lactobacilli and also of *L. delbrueckii* and *L. leichmannii*, glucose must be present in the medium at a concentration of 2% (Briggs, 1953). The aesculin test,

as reported by Rogosa *et al.* (1953), has proved unsatisfactory; but further work has shown that this test may be assessed either by a loss of fluorescence or by blackening of the medium in the presence of ferric ammonium citrate (Davis, 1955; Naylor & Sharpe, 1958). Tests for gas production depend on the utilization of relatively large concentrations of available carbohydrate and the use of large inocula from vigorous cultures (Gibson & Abd-el-Malek, 1945; Rogosa *et al.* 1953). It need hardly be said that correctly controlled water baths are indispensable for the determination of growth temperatures; but it is not generally appreciated that the temperature of unstoppered tubes in a 45° bath may be 2–3° lower at the surface of the medium. As for serological studies, three factors are obviously important: the production not only of specific sera representative of groups of micro-organisms but also of strongly reacting sera, and the avoidance of nonspecific haptenes (Sharpe, 1955*a,b*; Sharpe & Wheeler, 1957). In nutritional tests the development of physiologically balanced media is essential. It should not be assumed that since a little of a compound is good, more is better; or, that assay media designed for specific purposes can be widely applied. Work now in progress sharply underscores this point, since we have doubled the rate and extent of growth in widely used complete assay media by reducing the concentration of folic acid and other compounds to non-inhibitory levels. Certain organisms, *L. bulgaricus* in particular, may be prevented from growing altogether by small amounts of folic acid or of Cu, or be so strained in growth and metabolism that certain pathways of synthesis may be blocked. Our results are based on cultures which reached maximal growth in 18 hr with optical densities of 1 or higher in tubes 15 mm in diameter. The media and other details of methodology will be published elsewhere.

4. THE DIFFERENTIATION OF SPECIES

Before presenting our scheme for the species differentiation of the lactobacilli, we would reiterate what has previously been stated by Rogosa *et al.* (1953)—“Often it is forgotten that the determinations made in this and similar studies are of the characteristics of *bacterial populations*. In the absence of special techniques for the isolation and selection of mutants or special variants, what is determined is the *resultant mass character* of the total bacterial population.” With scrupulous attention to the matters of technique already discussed, extensive experience with individual strains has been gratifying since it is a rare occurrence indeed that consistent repetitive results are not to be obtained.

(a) *The subgenus Thermobacterium*

The differentiating characteristics of the thermobacteria are presented in Table 1. As the name *Thermobacterium* (Orla-Jensen, 1919) suggests, these homofermentative organisms are expected to grow at the elevated temperatures of 45° or higher. Rarely, there are exceptions to this; and what is worse is that certain organisms which are not thermobacteria, i.e. *L. casei* var. *rhamnosus*, sometimes *L. plantarum*, and probably others, can also grow at 45°. If a single growth test at 45° is depended on for a primary division of the homofermentative lactobacilli, confusion may result.

Fortunately, however, a constant differentiating characteristic is available since a *Thermobacterium* sp. which can grow at 15° has not yet been reported. Indeed, many strains do not grow at 20°. Thus, unlike the 45° growth test, inability to grow at 15° is a useful character because besides being constant there is also an adequate margin of dependability.

Orla-Jensen (1919) described three species, *Thermobacterium helveticum*, *Tbm. bulgaricum* and *Tbm. jugurti*. Of these, *Tbm. bulgaricum* (or *Lactobacillus bulgaricus*) has been very difficult to identify, principally because any lactobacillus isolated from such sour milks as yoghurt has been indiscriminately named *L. bulgaricus*. Nearly always, strains received as *L. bulgaricus* have been *L. helveticus* or *L. jugurti* (this appears to be the correct form of this specific name), or sometimes *L. lactis*. In 1953 Rogosa *et al.* probably too boldly designated *L. jugurti* as *L. bulgaricus*, because of the confused description in Bergey's Manual (Breed, Murray & Hitchens, 1948) and also because many of the strains received as *L. bulgaricus* were *L. jugurti*. But recently de Man (1956, 1960) has properly characterized enough strains which are truly

Table 1. *The differentiating characteristics of the species of the subgenus Thermobacterium**

Species of <i>Lacto- bacillus</i>	Colony type †	Growth at 15°	Serological groups	Acid in milk (%)	Lactic acid configuration	NH ₃ from arginine ‡	Aesculin cleavage	Amygdalin	Cellobiose	Galactose ‡	Lactose ‡	Maltose ‡	Mannitol	Mellobiose ‡	Raffinose ‡	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose ‡
<i>helveticus</i>	R	—	A	2.7	DL	—	—	—	—	+	+	+	—	—	—	—	—	—	—	+
<i>jugurti</i>	R	—	A	2.7	DL	—	—	—	—	+	+	—	—	—	—	—	—	—	—	+
<i>bulgaricus</i>	R	—	E	1.7	D(—)	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—
<i>lactis</i>	R	—	E	1.7	D(—)	—	—	—	—	+	+	+	—	—	—	—	+	—	+	+
<i>acidophilus</i>	R	—	?	0.3-1.0	DL	—	+	+	+	+	+	+	—	+	+	—	+	—	+	+
<i>leichmannii</i>	R	—	?	0	D(—)	±	+	+	+	—	±	+	—	—	—	—	+	—	+	+
<i>delbrueckii</i>	R	—	?	0	D(—)	±	—	+	—	+	—	±	—	—	—	—	—	—	+	—
<i>salivarius</i> var. <i>salivarius</i>	R	—	X	0.9	L(+) and DL	—	—	+	—	+	+	+	+	+	+	+	—	+	+	+
<i>salivarius</i> var. <i>salicinarius</i>	R	—	X	0.9	L(+) and DL	—	+	+	—	+	+	+	+	+	+	—	+	+	+	+

* All strains ferment glucose. None ferments adonitol, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, melezitose, sorbose, starch, xylose, α-methyl-D-glucoside, and α-methyl-D-mannoside.

L. helveticus, *L. jugurti* and *L. bulgaricus* may be variable or weak in fructose and mannose.

Dextrin is fermented by *L. leichmannii* and may be weakly fermented by *L. acidophilus* and *L. delbrueckii*.

Some strains of *L. acidophilus* ferment glycogen and *L. delbrueckii* may be variable or weak in mannose.

† R, rough colonies; in tomato juice agar without smooth colony inducing substances such as Tween 80 and acetate (Rogosa & Mitchell, 1950). §?, unknown; X, possible new group.

‡ ±, variable reaction; —, great majority of strains negative, sometimes weak or late positive.

L. bulgaricus as they are identical with the organism described by Orla-Jensen and since then we also have found a number of such strains. There is therefore now no doubt that *L. helveticus*, *L. jugurti* and *L. bulgaricus* are separate entities. But *L. helveticus* and *L. jugurti* may be biotypes or varieties of a single species, the only consistent difference being that *L. helveticus* ferments maltose whereas *L. jugurti* does not. Occasional strains of either species may ferment trehalose, and fructose and mannose may be fermented weakly. But otherwise they are remarkably alike: they both produce a very high mean acidity in milk, form DL-lactic acid, behave

identically in nutritional tests, and are also closely related serologically, having the same group antigen. *L. bulgaricus*, on the other hand, is more nearly related to *L. lactis* since both produce 1.7% of acid in milk, form D(-)-lactic acid and possess the same group antigen. However, they differ, as Table 1 shows, in four fermentations. *L. lactis* ferments maltose, salicin, sucrose and trehalose, whereas *L. bulgaricus* does not. The latter may also exhibit weak or negative fermentations of fructose and mannose. Kandler (pers. comm.) and Turner-Graff (pers. comm.) have differentiated certain species by the appearance of granules in cells stained with dilute (1:4) Loeffler's methylene blue. We also have found that whereas *L. bulgaricus* and *L. lactis* exhibit characteristic granules, *L. helveticus* and *L. jugurti* do not. This reinforces, by means of a simple technique, the relationships between these species.

The molecular configuration of the lactic acid produced distinguishes *L. acidophilus* from *L. leichmannii*, the former producing DL and the latter D(-)-lactic acid. If this were not determined it would be difficult to differentiate between them on the basis of fermentation tests alone. However, *L. leichmannii* does not produce any acid in milk, whereas *L. acidophilus* does. The inclusion of *L. leichmannii* in the low temperature homofermentative group in Bergey's Manual (Breed, Murray & Hitchens, 1948; Breed, Murray & Smith, 1957) is clearly incorrect. *L. delbrueckii* is quite distinct, as is evident from Table 1. *L. salivarius*, a species first described by Rogosa *et al.* (1953), is distinguished by a strong fermentation of mannitol and a wider range of carbohydrate fermentations. This species has two varieties, one of which hydrolyzes aesculin, ferments salicin and does not ferment rhamnose whereas the other does the opposite. Wiseman & Sarles (1957) have found *L. salivarius* to be very common in the chick, and Rogosa *et al.* (1953) and Rogosa & Disraely (1953) have shown that this is the predominating homofermentative species in the hamster.

Previously, Rogosa, Tittsler & Geib (1947), Rogosa *et al.* (1953) and Ford, Perry & Briggs (1957) had shown that nutritional requirements correlate very well with other species characteristics. Subsequent extensive and also recent experience with modified media, based on a much wider and contemporary knowledge, has amply confirmed this. Table 2 shows the differentiating nutritional responses of the thermobacteria

Table 2. *Some nutritional requirements of the subgenus Thermobacterium Orla-Jensen*

Species of <i>Lactobacillus</i>	Requirement* for					
	Thi- amine	Ribo- flavin	Pyri- doxal	Folic acid	Vitamin B ₁₂	Thymi- dine
<i>helveticus</i>	—	+	+	—	—	—
<i>jugurti</i>	—	+	+	—	—	—
<i>bulgaricus</i>	—	+	—	—	—	—
<i>lactis</i>	—	+	—	—	±	—
<i>acidophilus</i>	—	+	—	+	±	—
<i>leichmannii</i>	—	—	—	+	+ or s	—
<i>delbrueckii</i>	—	+	—	—	—	+
<i>salivarius</i>	—	+	—	+	—	—

*+, requirement; —, no exogenous requirement; ±, some strains exhibit erratic requirements under highly aerobic conditions, particularly in the absence of certain deoxyribosides; s, stimulatory.

which have been tested. *L. helveticus* and *L. jugurti*, which resemble each other closely, have the same nutritional requirements and are the only two species requiring pyridoxal. The close relationship of *L. bulgaricus* and *L. lactis* is seen here also, since they have similar nutritional requirements. An additional differentiation of *L. acidophilus* and *L. leichmannii*, beyond the already mentioned differences in titratable acid in milk and molecular configuration of lactic acid, is the ability of *L. leichmannii* to dispense completely with exogenous riboflavin without the rate or extent of growth and acid production from glucose being affected.

(b) *The subgenus Streptobacterium*

Table 3 presents the differentiating characteristics of the streptobacteria. These organisms, unlike the thermobacteria, grow at 15° and usually even at 6°. *L. plantarum* differs from *L. casei* in the amount and kind of lactic acid produced in milk, in not producing gas from citrate, in pentose and melibiose fermentations, in growth in Teepol (an anionic detergent comprising alkyl sulphates, Shell Chemical Co., Ltd.), in antigenic structure and in nutritional behaviour.

Table 3. *The differentiating characteristics of the known species of the subgenus Streptobacterium*

Species of <i>Lacto-bacillus</i>	Colony type	Growth at 15°	Serological group	Acid in milk (%)	Lactic acid configuration	Gas from citrate	Growth in 0.1% Teepol	Arabinose†	Lactose	Melibiose	Sucrose	Xylose†	Inulin†	Raffinose	Rhamnose†	Sorbitol	Sorbose†
<i>plantarum</i>	S	+	D	0.3-1.2	DL	—	+	± ¹	+	+	+	± ²	± ³	± ⁴	± ⁵	± ⁶	—
<i>casei</i> var. <i>casei</i>	S	+	B,C ⁸	1.2-1.5	L(+)	+	—	—	+	—	+	—	± ³	—	—	+	± ⁷
<i>casei</i> var. <i>rhamnosus</i>	S	+	C	1.2-1.5	L(+)	+	—	—	+	—	slow or —	—	—	—	+	+	+
<i>casei</i> var. <i>alactosus</i>	S	+	B,C ⁸	0	L(+)	+	—	—	—	—	+	—	± ³	—	—	+	± ⁷

* All strains hydrolyse aesculin and ferment amygdalin, cellobiose, galactose, fructose, maltose, mannitol, mannose, melezitose, salicin and trehalose. Some strains ferment dextrin and dulcitol. Some strains ferment α -methyl-D-glucoside and some strains of *L. plantarum* α -methyl-D-mannoside. None ferments adonitol, erythritol, glycogen and starch. † ±, majority of strains positive; ∓, great majority of strains negative, sometimes weak or late positive.

¹, 69% + in the U.S.A. (Rogosa *et al.* 1953) and 79% in the U.K. (Wheater, 1955).

², 100% — in the U.S.A. (Rogosa *et al.* 1953) and 87% in the U.K. (Wheater, 1955).

³, 97% of strains negative.

⁴, Rogosa *et al.* (1953) found all strains +, and Wheeler (1955) 77% +.

⁵, Rogosa *et al.* (1953) found all strains — or weak, and Wheeler (1955) found 59% negative.

⁶, Great majority +.

⁷, Rogosa *et al.* (1953) found 84% of 196 strains +.

⁸, Great majority of strains in group B.

The nutritional differences between the two species are presented in Table 4. One very frequent biotype or variety of *L. plantarum* does not require riboflavin, whereas all strains of *L. casei* do. However, it is the opinion of one of us (M.R.) that *L. plantarum*, as presently constituted, may not be homogeneous and may really comprise more than one variety if not species (Rogosa *et al.* 1953). Also, *L. casei* either has an absolute pyridoxal requirement, or is highly stimulated by it. Folic acid is also indispensable for *L. casei*.

Table 4. *Some nutritional requirements of the subgenus Streptobacterium Orla-Jensen*

Species of <i>Lactobacillus</i>	Requirement* for			
	Thiamine	Riboflavin	Pyridoxal	Folic acid
<i>plantarum</i>	—	±†	—	—
<i>casei</i>	—	+	+ or v.s.	+

* +, requirement; —, no exogenous requirement; v.s., very stimulatory.

† Rogosa *et al.* (1953) have described two varieties of *L. plantarum*, one of which requires riboflavin. One of us (M.R.) believes they may be different even in a species sense.

(c) *The subgenus Betabacterium*

Table 5 shows the differentiating characteristics of the heterofermentative lactobacilli of the subgenus *Betabacterium* Orla-Jensen. Unfortunately, the distinctions are not nearly as clear cut as with the homofermentative species. *L. fermenti* may be considered the one known species which is more thermophilic than the others. But here again, as in the case of the homofermentative thermobacteria, the really important distinction is the inability of *L. fermenti* to grow at 15°. Fermentation tests alone are not too helpful in differentiating *L. fermenti* from *L. brevis*, since many strains of *L. fermenti* ferment arabinose or xylose or both. *L. buchneri* characteristically ferments melezitose, while *L. cellobiosus* ferments cellobiose. *L. viridescens* is relatively nonfermentative on most substrates.

Although it appears that there are fewer characters by which to differentiate the heterofermentative lactobacilli, additional correlative data may be obtained from nutritional studies. Table 6 shows that unlike the homofermentative organisms the heterofermentative strains require thiamine: but it is also clear that *L. buchneri*, which was previously differentiated from *L. brevis* only by a melezitose fermentation,

Table 5. *The differentiating physiological characteristics of the heterofermentative species within the subgenus Betabacterium Orla-Jensen**

Species of <i>Lacto- bacillus</i>	Growth at 15°	Growth at 45°	Growth in 0.1% Teepol	Serological group	NH ₃ from arginine	Aesculin cleavage†	Arabinose†	Cellobiose	Lactose†	Mannitol	Melezitose	Melibiose†	α-methyl- D-glucoside†	Raffinose†	Salicin†	Sucrose†	Trehalose†	Xylose†	Mannose†
<i>fermenti</i>	—	+	—	F	+	—	±	—	±	—	—	±	±	±	—	±	±	±	±
<i>buchneri</i>	+	—	+	E	+	±	+	—	±	weak or —	+	+	+	+	—	+	—	±	—
<i>brevis</i>	+	—	+	E	+	±	±	—	±	weak or —	—	+	±	±	— or weak	±	—	±	—
<i>cellobiosus</i>	+	—	+	—	+	+	+	+	±	—	—	+	—	+	±	+	+	±	weak ±
<i>viridescens</i>	+	—	+	—	—	—	—	—	—	weak or —	—	—	—	—	—	±	±	—	+

* No species ferments adonitol, amygdalin, dextrin, dulcitol, erythritol, glycerol, glycogen, inositol, inulin, rhamnose, sorbitol, sorbose, starch, or α-methyl-D-mannoside. All ferment glucose and maltose and produce DL-lactic acid. † ±, majority of strains positive; ±, great majority of strains negative, sometimes weak or late positive.

L. fermenti: 79% arab. —; 77% xyl. —; 62% arab.-xyl. —; 90% mel. + raff. +; 87% treh. —; 90% α-meth.-D-gluc. —; sucrose 100% + U.S., 77% U.K.; 62% mannose +; 88% fructose +; 85% lactose +.

L. buchneri: 86% xylose —; 90% lactose +; 10% lactose weak; 100% α-meth.-D-gluc. +.

L. brevis: 32% lactose +; 68% lactose weak; 68% raffinose +, 32% weak; sucrose 88% +, 12% —; salicin 56% weak, 44% —; xylose 88% +, 12% —; α-meth.-D-gluc. 88% +, 12% —.

may be further separated by the nutritional data. Most strains of *L. buchneri* (Rogosa *et al.* 1953; Perry, 1958) require riboflavin, whereas *L. brevis* grows well in its absence. In addition, folic acid is not required by *L. buchneri*. In our experience with many modifications of media and a large number of strains, *L. brevis* is the only known heterofermentative lactobacillus for which folic acid is indispensable. *L. fermenti* and *L. cellobiosus* have a similar nutritional pattern, although they are different in other ways. *L. viridescens* may be stimulated by pyridoxal and folic acid.

Table 6. *The differentiating nutritional requirements of the heterofermentative lactobacilli of the subgenus Betabacterium Orla-Jensen*

Species of <i>Lactobacillus</i>	Requirement* for			
	Thiamine	Riboflavin	Pyridoxal	Folic acid
<i>fermenti</i>	+	—	—	—
<i>buchneri</i>	+	±	—	—
<i>brevis</i>	+	—	—	+
<i>cellobiosus</i>	+	—	—	—
<i>viridescens</i>	+	+	s	s

* +, requirement; —, no exogenous requirement; ±, c. 75% of strains exhibit requirement; s, stimulatory.

5. DISCUSSION

The serological knowledge of the group antigens of the genus (Sharpe, 1955*a,b*; Sharpe & Wheater, 1957) has been usefully applied to our present work: but there are anomalies. First, there are two antigenic groups of *L. casei*, and second, certain widely divergent species, such as *L. lactis*, *L. bulgaricus*, *L. brevis* and *L. buchneri*, share the group E antigen. Unfortunately a satisfactory group antiserum for *L. acidophilus* has not yet been prepared. But to balance this it now seems that we have succeeded in preparing a group antiserum for *L. salivarius*.

Certain other studies which may also be valuable in differentiating lactobacilli from each other and from other genera have not been mentioned. Among these is the chromatographic technique for the analysis of cell components, which has been dealt with by Cheeseman (1959). Goulden & Sharpe (1958) have already demonstrated the differentiating value of infrared spectroscopy.

Also, certain species mentioned in the literature have not been discussed. On the basis of the evidence (Orla-Jensen, Orla-Jensen & Winther, 1936; Sundman, Björkstén & Gyllenberg, 1959; Gyllenberg & Carlberg, 1958; Dehnert, 1957) we do not consider *Lactobacillus bifidus* to be a member of the genus. In addition, Cummins & Glendenning (1957) have shown that the cell wall composition of this organism is generically distinctive and unlike that of the lactobacilli. Kandler (pers. comm.), using a different technique of cell wall extraction (Kandler, Hund & Zehender, 1958) has confirmed these results. Also, strains of the common brewery organism *L. pastorianus*, when sent to us for examination, have invariably been *L. brevis* or occasionally *L. buchneri*. This again sharply points up the confusion which can result from depending too heavily on habitat as a taxonomic criterion. As to the type species *L. caucasicus* (Breed, Murray & Hitchens, 1948; Breed, Murray & Smith, 1957), a strain of which

is not extant, the evidence now makes it nearly certain that *L. lactis* and that organism are identical. Since the name *L. caucasicus* has priority, *L. lactis* appears to be superfluous and should be renamed *L. caucasicus*.

In this paper, the terminology employed, *Thermobacterium* Orla-Jensen, *Streptobacterium* Orla-Jensen and *Betabacterium* Orla-Jensen, was adopted partly for historical reasons: but another reason was the basic soundness of the physiological divisions made by Orla-Jensen (1919). In the 6th edition of Bergey's Manual (Breed, Murray & Hitchens, 1948) the key to the species of the genus *Lactobacillus* used this terminology and divided the genus in the same way. This nomenclature has been questioned (Tittsler *et al.* 1952; Breed, Murray & Smith, 1957) on the grounds that it is illegitimate. But although Orla-Jensen's terminology has been abandoned in the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957), it was found logical to maintain the subdivisions set forth by Orla-Jensen and in previous editions of Bergey's Manual itself. Also, the order of presentation of the descriptions of species in the 7th edition (1957) is essentially unchanged from that of the previous edition. The situation as to nomenclature and divisions of the genus is more complicated than would appear from the discussion in the 7th edition, and since this is a difficult problem requiring considerable discussion and individual attention we defer discussion until a more appropriate time. We, and certain other workers, will then offer specific proposals for adjudication to the Judicial Commission of the International Association of Microbiological Societies.

Finally, we wish to leave these impressions. It is essential to accumulate reasonably complete information about a strain in order to arrive at a correct classification. Techniques should be standardized and should permit optimal physiological activity. Variable reactions should not be the primary point of separation. We make this point again—that the study of individual strains of lactobacilli through the years has shown that they are remarkably constant in their characteristics. Orla-Jensen long ago observed this (1919, 1943). However, what we have presented applies to typical species reactions. Undoubtedly, there are intermediate organisms, conditioned by habitat or special conditions, and there are probably species which are not yet understood. Nature is ever challenging and will always present us with problems for the future.

6. SUMMARY

The genus *Lactobacillus* has been defined in terms of the most recent knowledge and the subgenera *Thermobacterium* Orla-Jensen, *Streptobacterium* Orla-Jensen and *Betabacterium* Orla-Jensen accepted. The authors' concept of species in the genus has been stated both in biological and statistical terms, and general methods for the study of species and the necessary standardization of certain methods have been discussed.

Morphology and colony form on certain media, the nature of the fermentation (homofermentative or heterofermentative), limiting temperatures for growth, serology and physiological tests such as total acidity in milk, molecular configuration of lactic acid, fermentation of carbohydrates, resistance to alkyl sulphates, production of gas from citrate and ammonia from arginine, and nutritional requirements in optimal media have been employed for the differentiation of species.

Among the organisms of the subgenus *Thermobacterium*, *Lactobacillus helveticus* and *Thermobacterium jugurt* Orla-Jensen are considered as probable varieties of a single species and *L. bulgaricus* is recognized as distinct from these as well as *L. lactis*. The type species of the genus is *L. caucasicus*. Since *L. lactis* appears identical with *L. caucasicus* and since the latter epithet has historical priority, *L. lactis* was superfluous when first named and therefore should be abandoned. *L. acidophilus*, *L. leichmannii*, *L. delbrueckii* and *L. salivarius* are also defined. The error in Bergey's Manual, of including *L. leichmannii* in the low temperature homofermentative group, has been corrected.

In the subgenus *Streptobacterium*, *L. plantarum* and three varieties of *L. casei* have been described. However, organisms which are either intermediate between *L. plantarum* and *L. casei* or species not yet clearly understood also exist.

For the heterofermentative organisms of the subgenus *Betabacterium*, the characteristics by which *L. fermenti*, *L. brevis*, *L. buchneri*, *L. cellobiosus* and *L. viridescens* may be differentiated have been presented. However, it is recognized that differentiation of species among the heterofermentative lactobacilli is more difficult and may be more uncertain than with the homofermentative lactobacilli. *L. pastorianus* is identical with other named species, such as *L. brevis* or *L. buchneri*.

The organism '*Lactobacillus bifidus*' is rejected as a lactobacillus because its properties are inconsistent with the genus definition.

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THE APPLICATION OF CHROMATOGRAPHIC TECHNIQUES FOR THE IDENTIFICATION OF SPECIES AND STRAINS OF LACTOBACILLI

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1. INTRODUCTION

THIS PAPER deals with the chromatographic study of the amino acids and peptides which occur free within the bacterial cell, and with the collation of the results for the differentiation of certain strains and species of the genus *Lactobacillus*.

The more detailed differentiation of micro-organisms is based upon their physiological characters, e.g. fermentation of sugars, production of gas, etc. Such characters are a reflection of metabolic processes occurring during growth. The accumulation of amino acids and peptides within the cell, whether due to anabolic or catabolic processes, is also in great part a reflection of the metabolism of the micro-organism.

2. TECHNIQUE

The technique used in the study of quantitative and qualitative differences in the free amino acid pool of micro-organisms was described by Mattick *et al.* (1956). This technique involves growing the organism under optimum growth conditions with subsequent harvesting and washing of the cells, followed by their extraction with dilute acetic acid. The extract is then spotted on to filter paper and subjected to two dimensional chromatography using the solvent system of Levy & Chung (1953).

It is of course most important that the chromatogram patterns should be accurately reproducible, and the technique must therefore be carefully standardized so that variations are minimized. If care is taken the reproducibility can be very good.

An example of the distribution of an artificial mixture of amino acids in this system is shown in Plate 1 (a). The first solvent used, in the horizontal direction on the print, was *n*-butanol-water-acetic acid (4:5:1 v/v), and the second solvent, in the vertical direction, was *m*-cresol-phenol (2:1 w/w) saturated with borate buffer of pH 8.3. The paper was also impregnated with the same buffer between the two solvent runs. Development of the amino compounds was with 0.2% ninhydrin in ethyl alcohol containing acetic acid and collidine. The amino acids shown in Plate 1 (a) are, starting from the top left corner and proceeding horizontally, aspartic acid (1), glutamic acid (2), diaminopimelic acid (3), lysine (4), serine (5), asparagine (6), glycine (7), threonine (8), glutamine (9), alanine (10), β -alanine (11), tyrosine (12), hydroxyproline (13), α -aminobutyric acid (14), histidine (15), arginine (16), β -aminobutyric acid (17), valine (18), methionine (19), the leucines (20), proline (21) and phenylalanine (22).

3. CHANGES IN THE FREE AMINO NITROGEN POOL DURING GROWTH

(a) Changes with age of culture

Several workers, for example Gale (1947) using *Streptococcus faecalis*, Pauletta & Defranceschi (1952) using *Mycobacterium tuberculosis* and Majumdar & Bose (1958) using *Bacillus subtilis*, have shown that qualitative and quantitative changes occur in the free amino nitrogen pool during growth. The speed with which these changes

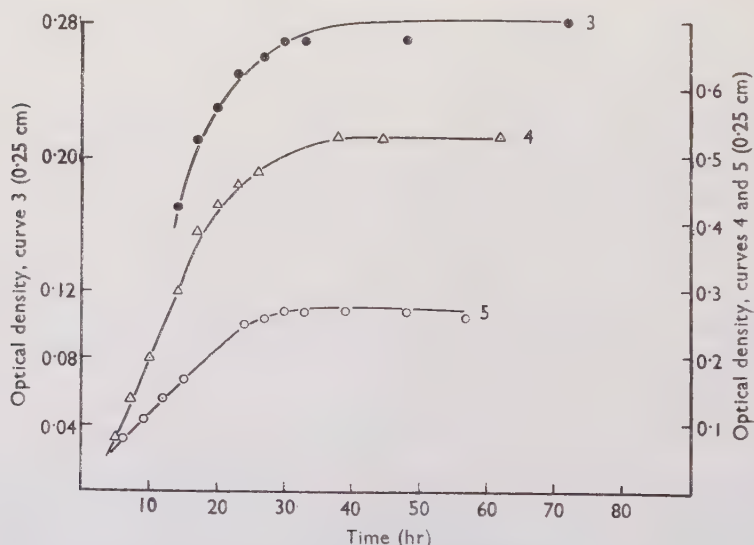


Fig. 1. The relation between period of growth and optical density.
 Curve 3, *Lactobacillus casei* A121.
 Curve 4, *Lactobacillus bulgaricus* B14.
 Curve 5, *Lactobacillus brevis* X2.

(From Berridge *et al.* 1957)

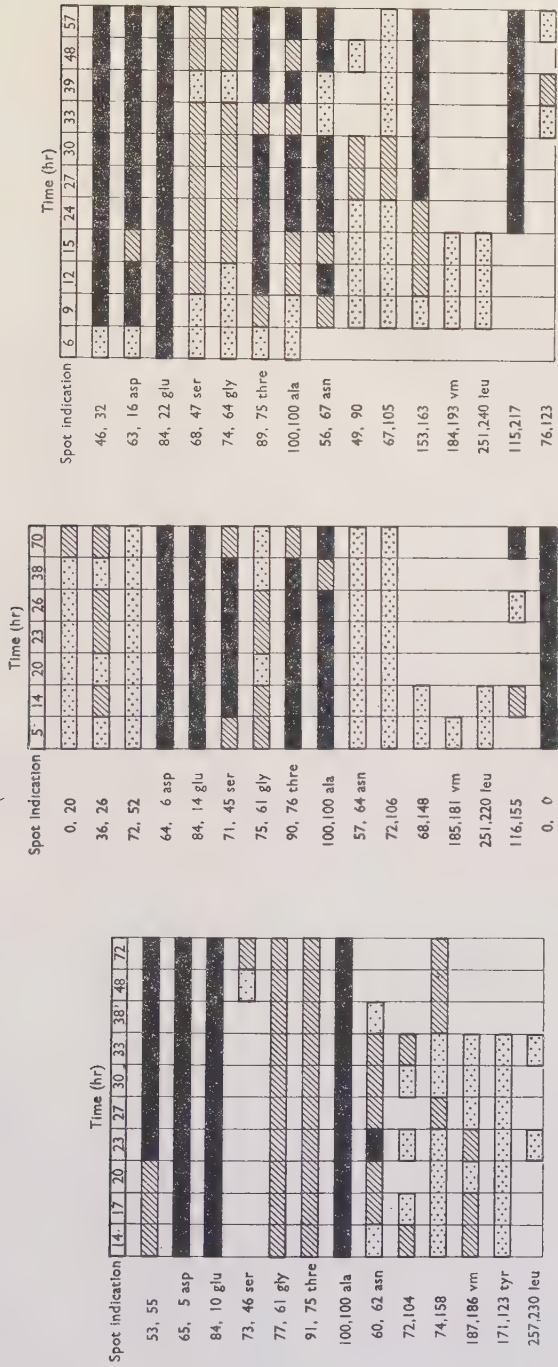


Fig. 2. The relation between period of growth and intensity of ninhydrin reaction.
Diagram 1: *Lactobacillus casei* A121.
Diagram 2: *Lactobacillus bulgaricus* B14.
Diagram 3: *Lactobacillus brevis* X2.
The intensities of the spots are indicated by: stippled, weak reaction; diagonal lines, medium reaction; solid, strong reaction; blank, negative.
(From Berridge *et al.* 1957)

take place appears to vary in different genera, perhaps because of differences in cell wall permeability or in the rate of metabolism. However, the rate of change of the internal amino acid pool does not appear to be significant for the species of lactobacilli examined. Berridge *et al.* (1957) demonstrated that a stable pattern can be obtained at the end of the logarithmic phase of growth, and it can be seen from Figs. 1 and 2 that the main variations in the patterns occurred during the early stages of growth and also several hours after the end of active growth. Fig. 1 shows the growth curves of strains of *Lactobacillus casei*, *L. bulgaricus* and *L. brevis*, all the cultures having been inoculated with 2% by volume from a 24 hr broth culture. Fig. 2 shows the presence, absence and intensities of the different ninhydrin positive spots, each horizontal bar corresponding to one spot. The pairs of numbers on the left of each diagram are the distances (mm) of the spots from each axis. The most stable patterns occurred 26-30 hr after inoculation.

(b) *Changes with medium and temperature of incubation*

The effects of medium and temperature of growth have also been studied. The *L. casei-plantarum* group showed little difference in the patterns obtained when grown in Yeastrel glucose broth (YGB) or tomato glucose broth (TGB), although growth in the latter was usually better; TGB was therefore used as the standard medium throughout the work. As an example of constancy within the *L. casei-plantarum* group, the chromatograms from two strains of *L. casei*, one grown in YGB and the other in TGB, are shown in Plate 1, (b) and (c). Both exhibit the characteristic pattern for *L. casei* strains, and the individual patterns show only slight strain differences. In this work, all the strains were grown at their optimum temperature; but it would have been better to have standardized the growth temperature at 30°, as the patterns of a single strain grown at both 30° and 37°, even when harvested at the same physiological age, may differ in detail. In Plate 1, (d) and (e), the patterns obtained from a strain of a high optimum temperature heterofermentative *Lactobacillus* grown in TGB at 37° and 30° are shown. It is evident that the major differences between them are the presence of tyrosine in the extract from the culture grown at 37°, and less serine and glycine in the culture grown at 30°.

4. THE SEPARATION OF *L. CASEI* AND *L. PLANTARUM*

Seventy-three strains of the *L. casei-plantarum* group were examined by Cheeseman *et al.* (1957) and separated into the two species by means of six features, which are shown in Table 1. Of the 73 strains 10 were not characterized absolutely as *L. casei* or *L. plantarum* and are not included in the Table. Only two strains of each species were atypical on two features; of these, however, the two strains of *L. plantarum* were also atypical biochemically, as they exhibited catalase activity. There did not appear to be any correlation between the chromatogram results and the serological groups B and C of *L. casei*. Nevertheless, further subdivision of both species on chromatogram pattern was possible and, indeed, several strains produced characteristic patterns. As an example of the patterns, the chromatograms from one strain of each of *L. plantarum* and *L. casei* are shown in Plate 2, (a) and (b), in which the six

Table 1. A summary of the distinguishing characteristics on chromatograms of 63 strains of *L. casei* and *L. plantarum*

Property	Nos. of strains of			
	<i>L. casei</i>		<i>L. plantarum</i>	
	Typical	Atypical	Typical	Atypical
Origin				
— in <i>casei</i>				
+ in <i>plantarum</i>	29	0	30	4
α -Aminobutyric acid				
— in <i>casei</i>				
+ in <i>plantarum</i>	29	0	30	4
Proline				
— in <i>casei</i>				
+ in <i>plantarum</i>	27	2	34	0
Asparagine				
— or faint in <i>casei</i>				
strong + in <i>plantarum</i>	26	3	33	1
Ratio glycine: threonine				
≥ 0.8 in <i>casei</i>				
< 0.8 in <i>plantarum</i>	20*	8	24*	5
Ratio 50,70: alanine				
> 1.0 in <i>casei</i>				
< 1.0 in <i>plantarum</i>	27*	1	27*	2

* Excluding 5 strains of *L. plantarum* and 1 strain of *L. casei* which were not examined quantitatively.

characterization features can be seen. The strong asparagine, aminobutyric and proline spots are present in the *L. plantarum* pattern and the differences in the lysine region (50, 70): alanine ratios and the glycine: threonine ratios are also apparent. The apparently positive origin in the *L. casei* chromatogram (b) is due to a brown discoloration and not to the normal blue coloration obtained with ninhydrin.

5. THE SEPARATION OF HETEROFERMENTATIVE LACTOBACILLI

Ninety-one strains of heterofermentative lactobacilli were also examined by Cheeseman & Silva (1959) and similarly divided by means of chromatogram features, in this case into two main groups corresponding to the high and low optimum temperature groups. An example of the pattern of a strain from each of these groups is shown in Plate 2, (c) and (d), in which two of the features used can be seen, namely the presence of glutamine and the weak lysine region in the chromatogram from the *L. brevis* strain (c) and the absence of glutamine and the very strong lysine region in that from the *L. fermenti* strain (d).

The results from the heterofermentative strains were analyzed mathematically in order to obtain an unbiased system of classification (Cheeseman & Berridge, 1959). The intensities of the spots from the chromatograms were assessed visually using scores of 1 to 7, which represented amounts of approximately 1.5–100 μ g of amino acids/cm². This form of scoring was condensed for the purpose of analysis to three categories: weak, scores up to 2; medium, scores over 2 and up to 5, and strong, scores over 5. The data, suitably arranged, were analyzed by an electronic computer,

using the methods described by Sneath (1957). The similarity values (S values) obtained did not however permit any clear division into groups. A family tree (Fig. 3) prepared from these S values shows that the heterofermentative lactobacilli form a spectrum of strains, from those which are very similar to most others (e.g. nos. 20 and 36) to those which are quite different from all others (e.g. nos. 1, 32 and 68).

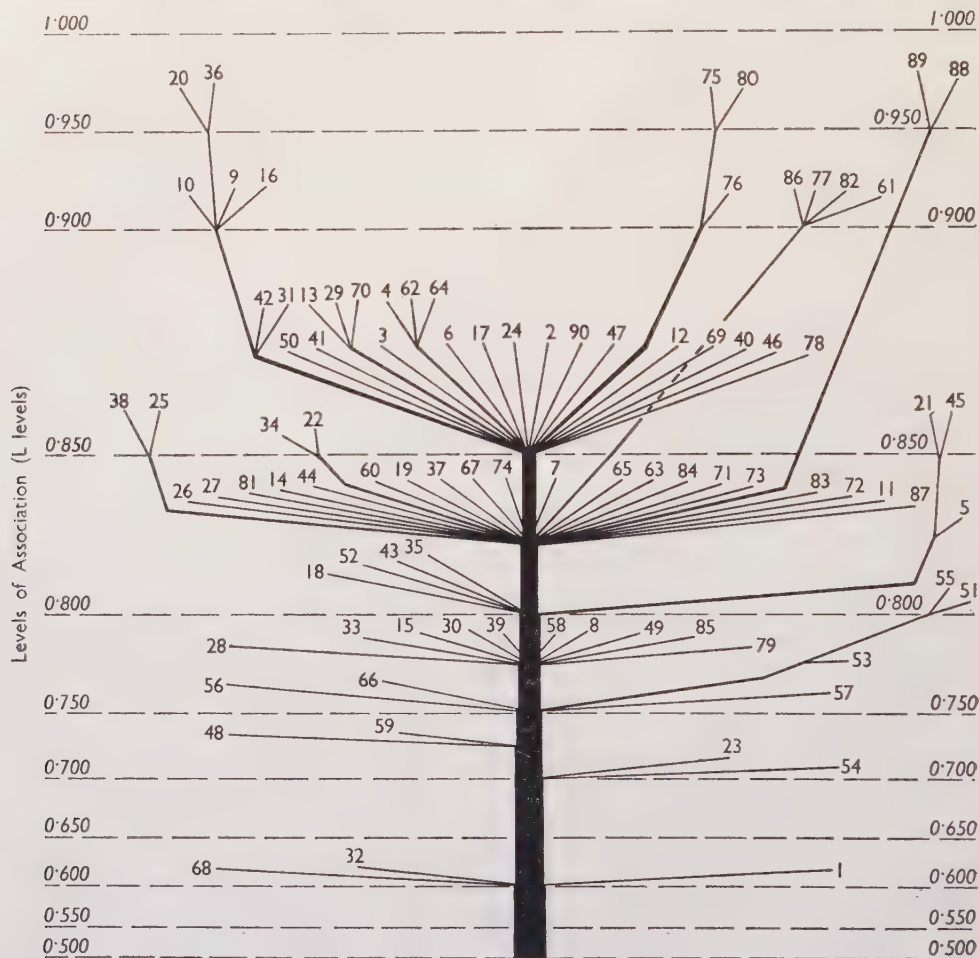


Fig. 3. A family tree of the strains of heterofermentative lactobacilli, arranged from the similarity values obtained by electronic computation. The horizontal broken lines indicate the level of similarity value.

(From Cheeseman & Berridge, 1959)

The horizontal lines on the Figure indicate the degrees of similarity shared by the groups of strains which fuse at certain points. However, as the arrangement of the cultures in the family tree is only two dimensional it cannot reproduce the complete relationships revealed in the full table of S values.

6. THE RELATIONSHIP BETWEEN *L. LACTIS* AND OTHER LACTOBACILLI

Strains of *L. lactis*, *L. leichmannii*, *L. delbrueckii* and *L. salivarius* have also been studied (Silva & Cheeseman, 1959), and the patterns obtained compared with those from the *Lactobacillus casei-plantarum* group. The comparison of eight different features is shown in Table 2. *L. plantarum* differed from the other strains in the presence of proline and aminobutyric acid, and the ratio lysine region:alanine. *L. salivarius* resembled the *L. casei-plantarum* group in glutamic acid:aspartic acid ratio and the absence of glutamine, and *L. casei* in lysine region:alanine ratio and in the absence of asparagine; but it was similar to the *L. lactis-leichmannii-delbrueckii* group in having a positive origin and in alanine:threonine ratio. The weight of

Table 2. *A comparison of certain chromatographic features of L. plantarum and L. casei with those of L. salivarius, L. leichmannii, L. lactis and L. delbrueckii, and their interrelationships*

(From Silva & Cheeseman, 1959)

Chromatogram feature	<i>L. plantarum</i>	<i>L. casei</i>	<i>L. salivarius</i>	<i>L. leichmannii</i>	<i>L. lactis</i>	<i>L. delbrueckii</i>
Origin	+ (97%)§	—	+	+ (71%)	+ (73%)	+
App. ratio glutamic: aspartic acids	≥2	≥2	≥2	1†	1 (87%)	1
Ratio lysine region: alanine	<1	>1	>1	>2	>2	>2
Proline	+	— (93%)	—	—	—	—
Aminobutyric acid	+ (97%)	—	—	—	—	—
Asparagine	+ (97%)	— (90%)	— (75%)	+ (86%)	+ (93%)	+
Glutamine	mainly —ve	mainly —ve	—*	+	+ (67%)	+
Ratio alanine: threonine	>1	>1	<1	LE3, LE6, LE7 >1, rest <1	<1‡	variable

* Strain 8G variable. † Except strains LE9 and LE10, which had only traces of aspartic acid.

‡ Except strains of group 5.

§ The bracketed figures indicate the percentages of the strains with the features indicated.

evidence therefore suggests that *L. salivarius* is closer to *L. casei* than to *L. lactis*. However, the detailed results obtained from the examination of 45 strains of *L. lactis* suggest that this species is heterogeneous. These strains were subgrouped by chromatogram pattern differences and it appears likely that some strains supposed to be *L. lactis* are in fact closely related to other species. Bottazzi (1958) examined the patterns from a number of strains of *L. bulgaricus*, *L. acidophilus*, *L. helveticus* and *L. lactis* and succeeded in differentiating *L. lactis* from the others by means of several features; however, he also found some strains of *L. lactis* which had intermediate features, or were similar to the other species. It was not possible to differentiate between *L. bulgaricus* and *L. helveticus*, but these could be distinguished from *L. acidophilus*. Examples of the chromatogram patterns of one strain each of *L. lactis*, *L. bulgaricus*, *L. acidophilus* and *L. helveticus* are shown in Plate 2, (e), (f), (g) and (h);

differences in the patterns are clearly visible. The difference in alanine: threonine ratio, the strong origin, and the presence of what is possibly diaminopimelic acid are all clearly shown.

7. APPLICATION TO PRACTICAL PROBLEMS

Experiments involving the routine examination of lactobacilli from two Cheddar cheese have also been undertaken (Cheeseman, 1960), the advantage being that the chromatogram technique made it possible to examine between 60 and 80 cultures in the course of a week. The lactobacilli were isolated on acetate agar (Mabbitt & Zielinska, 1956), checked by microscopic appearance, catalase production, growth at 15° and at 45°, growth in 1% Teepol and gas production in Gibson's agar, and separated into groups on the basis of the similarity of their chromatogram patterns. The variation between strains within each group was slight. The groupings, and the

Table 3. *The distribution of lactobacilli in two Cheddar cheese**

Type of pattern	Group	No. of strains from cheese	
		A	B
Like the low optimum temperature group of heterofermentative bacilli	1 { (a)	3	1
	(b)	0	1
Like the intermediates of the <i>L. casei-plantarum</i> group	2	41	28
	3	10	18
Like <i>L. plantarum</i>	4	1	6
	Totals	55	54

* Made on consecutive days.

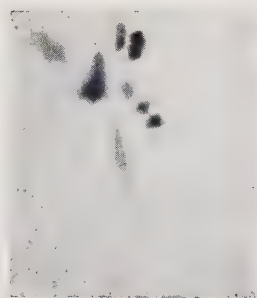
numbers of strains from each cheese, are shown in Table 3. The majority of the strains isolated fell into three groups with patterns similar to those previously encountered in the *L. casei-plantarum* group, but a few had patterns like those of the low optimum temperature group of the heterofermentative lactobacilli. The normal biochemical characterization tests were applied to some of the strains from each group, and the results agreed well with the chromatogram groupings.

8. CONCLUSION

The chromatographic examination of several species of lactobacilli has shown that within most species the strains show considerable heterogeneity; but this is perhaps to be expected. Division into species has of necessity been arbitrary, because emphasis has had to be placed on a few biochemical characters. Whether it will be possible to arrange any comprehensive system of classification for lactobacilli based upon chromatogram patterns will depend upon the results obtained when the work is extended to the other species not yet examined, and on whether the features upon which this system may be based prove really stable. Nevertheless, although the work is still incomplete and much more has to be done before the value of the method as an aid in the



(a)



(b)



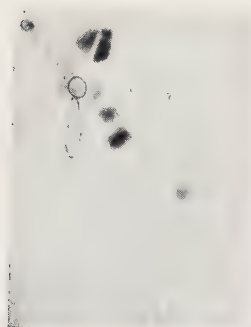
(c)



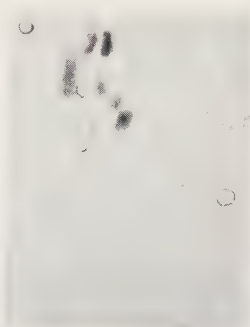
(d)



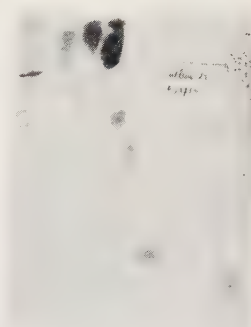
(e)



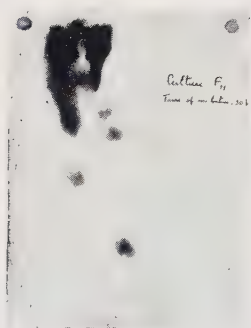
(a)



(b)



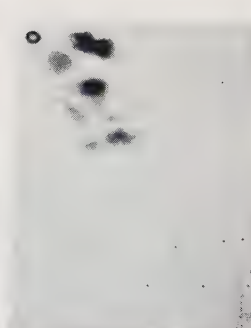
(c)



(d)



(e)



(f)



(g)



(h)

taxonomy of the genus *Lactobacillus* can be assessed, the present evidence indicates that the technique could be of use to the bacteriologist either in routine work or for more specific problems.

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EXPLANATION OF PLATES

PLATE 1. Examples of chromatograms.

- (a) A standard mixture of amino acids.
- (b) *L. casei* C2 grown in Yeastrel glucose broth.
- (c) *L. casei* C1 grown in tomato glucose broth.
- (d) *L. fermenti* CD2 grown at 37° in tomato glucose broth.
- (e) *L. fermenti* CD2 grown at 30° in tomato glucose broth.

PLATE 2. Examples of chromatograms.

- | | |
|----------------------------|-------------------------------|
| (a) <i>L. plantarum</i> | (e) <i>L. lactis</i> L4 |
| (b) <i>L. casei</i> | (f) <i>L. bulgaricus</i> B17 |
| (c) <i>L. brevis</i> V7 | (g) <i>L. acidophilus</i> A15 |
| (d) <i>L. fermenti</i> F11 | (h) <i>L. helveticus</i> A45 |

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LACTOBACILLI OF THE HUMAN MOUTH

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1. INTRODUCTION

LACTOBACILLI are regularly found in the human mouth, intestine and vagina. Each of these sites supports a complex and characteristic microbial flora; and that of the mouth contains representatives from a large number of genera, especially of Gram-positive bacteria. Among these, the lactobacilli have for long received a great deal of attention, although it is only recently that they have become probably the best understood members of the oral flora.

Widespread and important as are the lactobacilli in many fields, it is only in the mouth that they have received the honour (bacteriologically speaking) of being regarded as pathogenic. Progress in the rationalization of their systematics has been painfully slow, despite all the efforts of dental and other workers, largely due to the practical difficulties of isolation and culture. Now that so much progress has been achieved, it is odd, or gratifying (depending upon one's point of view) to record that the oral lactobacilli are currently regarded by many dental workers as an interesting but unimportant group. In the vagina and gut, as in other sites, the lactobacilli are generally viewed with a benevolent eye and it is only in relation to the unique tissue known as dental enamel that man has found the lactobacilli other than beneficial. The concept of their pathogenicity was never easy to accept.

The soft and hard dental tissues are each susceptible to disease, the former to periodontal disease, ranging from slight inflammation to acute ulceration of the delicate gingival tissues, and the latter to dental caries, a unique disease of a unique

tissue. Both diseases involve bacteria and in each case the bacteria found in active lesions appear to be no different from those found in completely healthy mouths. Several such diseases are known. Rósebury (1958) calls them 'endogenous diseases', their characteristic being sensitivity to subtle changes in the environment which induce apparently harmless commensal micro-organisms to invade and create a pathological disturbance. In the case of caries, the Gram-positive, acid producing bacteria bring about decalcification of the teeth, whereas in periodontal disease a predominantly Gram-negative flora causes ulceration of the gingiva. In view of the grossly different floras of these two diseases, and the differences in the ecological factors which appear to encourage them, it is not particularly surprising that the two diseases very rarely occur together.

2. THE SIGNIFICANCE OF LACTOBACILLI IN DENTAL CARIES

Dental caries is defined as a disease of the dental hard tissues in which the lesion commences on the external tooth surface and progresses inward toward the pulp. The tooth surface, that is, dental enamel, is a highly resistant, highly calcified tissue of characteristic structure, containing only about 1% of organic material resembling keratin. Almost the only substances greatly deleterious to enamel are acids, and it has been generally accepted for many years that the acid fermentation by oral bacteria of carbohydrate food debris collected in tooth crevices and between teeth constitutes a major, if not the only, attack mechanism in dental caries. In certain points, however, this theory of caries is inadequate and many others have been proposed either as sole mechanisms or as adjuncts to the acidogenic theory. Most of these have proved equally or more difficult to substantiate, but two methods are known whereby caries may be curtailed: first, by restriction of intake of certain types of carbohydrate materials in the diet, and, second, by the addition of fluoride to drinking water. It is significant that both appear to operate by restricting acid attack, the former by reducing oral acid production and the latter by increasing the resistance of the enamel to acids.

Probably the first indication of a relationship between oral lactobacilli and dental caries was the finding by Goadby (1903) of an organism which he called '*Bacillus necrodentalis*' as a common inhabitant of carious dentine. The general desire to define more accurately the cause of caries crystallized between 1915 and 1930 around the lactobacilli. Howe & Hatch (1917) found lactobacilli regularly in carious lesions and the qualitative relationship between these organisms and caries incidence was much studied by Bunting (1928), who wrote "... the presence or absence of *Bacillus acidophilus* in the mouth constitutes a definite criterion of the activity of dental caries that is more accurate than any clinical estimation can be . . ."

With the discovery of suitable selective media the well-known '*Lactobacillus acidophilus* count' test (Rodriguez, 1930; Hadley, 1933) was evolved and a quantitative relationship between lactobacilli and caries was, and continues to be, regularly found (Jay *et al.* 1936; Green & Dodd, 1956a; Goldsworthy & Spies, 1958). This quantitative relationship has latterly come to be recognized as a guide to caries susceptibility rather than as direct evidence of the etiology of caries, and recent work (Lilienthal, 1956;

Sullivan & Goldsworthy, 1958) indicates that the oral lactobacillus count is actually controlled by, and directly related to, the dietary intake of carbohydrates. A somewhat hypothetical explanation of this relationship has been proposed recently (Lightfoot & Coolidge, 1959); but there is no evident reason why a simple theory of direct nutritional selection could not equally well account for the phenomenon.

Numerically the lactobacilli are minor members of the oral flora; saliva in caries-free mouths yields counts of 10^2 – 10^4 /ml, and in caries-active mouths 5×10^4 – 10^6 /ml, while the total salivary bacterial counts may range from 5×10^7 – 5×10^9 /ml. With the onset of caries, not only do the lactobacilli in the saliva increase in numbers, but also, and more significantly, their incidence in the bacterial films on the surfaces of teeth, the so-called dento-bacterial plaque, increases prior to cavity formation at a particular site (Hemmens *et al.* 1946; Green, Dodd & Inverso, 1957). Also, they are almost universally found in carious lesions, especially in enamel and deep dentinal caries (Burnett & Scherp, 1951), though other organisms are always present, as elsewhere in the mouth. These observations upon the incidence of lactobacilli, together with their known metabolic potential, form the basis for the theory that they are the etiological agents of caries. It has however proved a difficult theory to substantiate and, as already pointed out, it may simply be that the conditions which encourage caries also encourage the growth of lactobacilli. The actual significance in the various processes of such factors as the aciduric nature of lactobacilli has also been questioned and it has been shown on various occasions that other oral bacteria can at least assist in the carious process (e.g. Pigman *et al.* 1957). It therefore seems most reasonable at present to keep an open mind upon the subject of caries etiology.

3. THE ORAL LACTOBACILLI

(a) *Relationships to lactobacilli from other environments*

Moro (1900) and Tissier (1900) independently described bacteria of the *Lactobacillus* type from the infant intestine. Both described one type, for which the name *Bacillus acidophilus* (Moro) was generally accepted, and Tissier also described a second type which he called *Bacillus bifidus*. The similarity between these organisms and others from dairy sources was recognized by White & Avery (1909), and this group formed the nucleus of the genus *Lactobacillus* set up by Winslow *et al.* (1920).

From 1900 onwards the names most commonly used for strains from the human body have been the 'Boas-Oppler bacillus' for intestinal strains, 'Doderlein's bacillus' for vaginal strains and *B. acidophilus* for oral, intestinal and vaginal strains; but the name *acidophilus* has in fact been used as a general term for all parasitic lactobacilli of the human body which do not resemble *B. bifidus* (Davis, 1956a). As stated above the name was originated by Moro (1900) for intestinal strains and in none of the seven editions of Bergey's Manual is the fact that lactobacilli occur in the human mouth recognized, except casually in the appendix to the genus.

Exactly when the oral strains began to be called *acidophilus* is not clear, but Kligler (1915) isolated from the human mouth 58 strains which he called *B. acidophilus*, and Rahe (1918) recorded that *B. acidophilus* and the gas producing *B. acidophil-aerogenes* occurred with almost equal frequency in saliva and in the gut. From these

studies two important points emerge: first, that the name *B. acidophilus*, subsequently modified to *L. acidophilus*, was firmly applied to the oral lactobacilli and used for the next thirty years, in spite of strong indications that several species were present; and second, that the early discovery of the occurrence of gas-forming lactobacilli in the human body was ignored until 1952, when Tilden & Svec once more brought the heterofermentative lactobacilli into dental bacteriology.

There have been other equally unfortunate episodes in the study of parasitic lactobacilli. Thus, for example, although there was considerable controversy for many years regarding the similarity between oral and intestinal strains, the practice of intestinal lactobacillus therapy, for which there was a certain vogue early in this century following the suggestion by Metchnikoff (1910) that a diet of fermented milk (i.e. yoghurt) improved health and prolonged life, resulted in a situation in which *L. acidophilus*, which soon replaced *L. bulgaricus* as the proposed therapeutic agent, was both being condemned by dentists as the cause of caries and lauded by dieticians as a cure for all ills. This difference of opinion led to redoubled efforts to classify the oral and intestinal lactobacilli and produced some very useful work, which culminated in the excellent studies directed by R. W. Harrison (e.g. Harrison & Opal, 1944); this foreshadowed the present classification of parasitic lactobacilli. It is now known that those who found the oral lactobacilli to be identical with those in the intestine and those who found them quite different were both partly right and partly wrong. The lactobacilli from both these sites, and also those from the vagina, are not habitat-specific species as was once imagined, but consist of representatives of various species, some of which, for example *L. acidophilus*, occur in all three sites.

With the general decline in the belief in lactobacilli as the cause of caries in favour of a more general acid-attack theory, and with the knowledge that lactobacilli of species other than *L. acidophilus* are more common in the mouth, there has been a slight revival of interest in acidophilus therapy. For example, a report of the apparently successful treatment of one form of periodontal disease has been published in Canada. Possibly related to this is the report that collagenase, an enzyme which may play a role in periodontal disease, is inhibited by some action, the nature of which is not known, of other members of the oral flora (Thonard & Scherp, 1958). This may also be correlated with the finding by Torrey & Kahn (1923) that lactobacilli can successfully inhibit the proteolytic activity of clostridia by acid action. Whether the proteolytic enzymes produced by oral bacteria are similarly affected by acids is not known.

(b) *Species found in the human mouth, and their sources*

The human oral lactobacilli include the homofermentative species *L. acidophilus*, *L. casei* and *L. plantarum*, and the heterofermentative species *L. fermenti*, *L. buchneri* and *L. brevis* (Rogosa *et al.* 1953; Grubb & Krasse, 1953; Davis, 1955, 1956a). In addition to these well-known species, three new ones have also been described from oral material: *L. salivarius* (Rogosa *et al.* 1953) and *L. melibiosus* (Featherstone, 1959), both of which are homofermentative, and *L. cellobiosus* (Rogosa *et al.* 1953) which is heterofermentative. Apart from the three latter species, which further

work may or may not prove to be specifically parasitic types, only *L. acidophilus* and a variant of *L. brevis* (Hayward & Davis, 1956) have so far been described almost exclusively from parasitic sources.

Although most *Lactobacillus* species appear to be primarily saprophytic and many of the common oral types no doubt enter the mouth in foodstuffs, it is not easy to decide what their primary habitat may be. The fact that lactobacilli are to be found in the air and on growing vegetation does not prove that they are primarily saprophytic, but simply indicates that certain *Lactobacillus* types can live under a variety of conditions. It is true that Hunt & Rettger (1930) isolated lactobacilli from soil and stored cereals, but it seems probable that they really came from animal faeces.

Table 1. *The distribution of Lactobacillus species in terms of numbers of strains isolated from various sources*

Type and species	(Data from Hayward, 1957)				
	Nos. of strains isolated from				
	Human saliva (16)*	Human vagina (31)*	Animal faeces (21)*	Cheese (29)*	Silage (8)*
Homofermentative					
<i>L. casei</i>	276	—	17	247	3
<i>L. plantarum</i>	21	—	—	52	21
<i>L. salivarius</i>	23	3	4	—	—
<i>L. acidophilus</i>	70	4	27	6	—
Unidentified	10	1	20	32	16
Heterofermentative					
<i>L. fermenti</i>	201	10	51	35	—
<i>L. brevis</i> (parasitic)	28	—	8	—	—
<i>L. brevis</i> (saprophytic)	14	—	—	177	11
<i>L. buchneri</i>	28	—	—	2	17
Unidentified	2	—	10	—	2

* Nos. of samples in brackets.

But whereas certain species appear to be more adaptable than others to new environments and occur with apparently equal frequency in material from animal and vegetable sources, others occupy restricted habitats and do not appear to survive well outside them. Hayward (1957) investigated the distribution of species and obtained the results shown in Table 1. He also noted that many of his atypical, unidentified strains resembled either *L. plantarum* or *L. brevis*, and this is of interest in view of the wide distribution of these species and the fact that some very atypical variants of them, such as motile and pigmented forms, have been reported (e.g. Harrison & Hansen, 1950; Costilow & Humphreys, 1955; Millis *et al.* 1959).

To gain a better idea of the incidence of various *Lactobacillus* spp. in the mouth, it is interesting to compare the results obtained by Featherstone (1959) in a survey of the mouths of Australian aborigines with those of workers using European (Hayward & Davis, 1956) and American material (Rogosa *et al.* 1953). The data given in Table 2 show that considering the difference in the methods used to obtain the American

Table 2. *The incidence of Lactobacillus species in three human communities*(Data from Featherstone, 1959; Hayward & Davis, 1956; and Rogosa *et al.* 1953)

Species	Incidence of positive mouths (%)		
	American (130)*	Italian urban (66)*	Australian aborigine (22)*
<i>L. casei</i>	59	45	4
<i>L. plantarum</i>	5	13	13
<i>L. salivarius</i>	6	7	36
<i>L. acidophilus</i>	21	49	13
<i>L. fermenti</i>	45	45	50
<i>L. brevis</i>	17	25	22
<i>L. buchneri</i>	10	12	0

* Nos. of mouths in brackets.

and Italian isolates there is relatively good agreement between the results for most species, except perhaps *L. acidophilus*. The species incidence in the mouths of aborigines does however appear to be rather different, notably in the low incidence of *L. casei* and the high incidence of *L. salivarius*. Whether this difference is due to different experimental handling or, as seems probable, is a consequence of dietary differences between this group and the other two is uncertain: but it is noteworthy that most primitive peoples, whose standard of oral hygiene is poor, appear to be more prone to periodontal disease than to caries.

4. THE DIFFERENTIATION OF SPECIES

No detailed survey has apparently been made of the distribution of *Lactobacillus* spp. in the various microhabitats of the mouth. With a view to facilitating such work we have experimented with a few methods which may allow early selection or rapid diagnosis of various species. Although incomplete and not as yet entirely satisfactory, the results may be of interest to other workers who encounter this problem.

The tests were carried out on five strains of each of eight species. *L. plantarum* was the most resistant to penicillin, with *L. brevis* (saprophytic) and *L. buchneri* also fairly resistant; the other species were inhibited by 1 u/ml in surface cultures on agar. It has previously been reported (Rogosa, Mitchell & Fitzgerald, 1950) that heterofermentative species, particularly the saprophytic *L. brevis* type (Hayward, 1957), are tolerant of penicillin. Sodium fluoride at 500 p/m inhibited the parasitic *L. brevis* strains, but the other species all showed some growth (see Shiota, 1956). Benzoic acid, sorbic acid and thallous acetate had no selective action. Casein-mannitol agar incubated at 20° allowed selection of *L. casei* and *L. plantarum*, and so did casein-aesculin agar at 20°. The mannitol agar at 45° supported only *L. salivarius* strains. Other tests, such as the reduction of triphenyltetrazolium chloride, gave variable results, as indicated by Burnett & Schmidt (1955).

The possible use of selective media based upon the known nutritional needs of the lactobacilli may be of value, but beyond testing the inhibition of *L. fermenti*

by neopyrithiamine, an analogue of thiamine (Wilson & Harris, 1949), we have not investigated this possibility.

In this field of species diagnosis the use of cell and colony morphology is of interest (Davis, Bisset & Hale, 1955; Davis, 1956*b*). The morphological features of *Lactobacillus* species have been frequently described and in general the descriptions agree with each other fairly well, the chain forming type of *L. casei* (Orla-Jensen's '*Streptobacterium*') and the long rigid cells of the *L. bulgaricus-acidophilus* group being particularly well known (Ross & Deutsch, 1957).

5. SYMBIOSIS, ANTAGONISM AND CONTROL

(a) *Symbiosis*

The existence of both symbiotic and antagonistic relationships between the micro-organisms of the oral flora has received considerable attention, primarily with regard to their possible roles in aiding or inhibiting oral disease processes. Probably the best known of these is the apparent relationship between lactobacilli and the yeast-like fungi of the genus *Candida*. *Candida albicans* is a potentially pathogenic organism, but it exists non-pathogenically in about 40% of mouths, and is the commonest oral fungus. Almost as soon as it was recognized that the lactobacilli were concerned in the incidence of dental caries, a similar relationship was also reported between *Candida* and caries (Bunting & Palmerlee, 1925; Rosebury & Waugh, 1939). Fosdick & Hansen (1936) suggested that oral lactobacilli and candidas live in close symbiosis, and that such associations yield more acid end-products than either organism alone, and may consequently be important factors in the production of caries. Their results from such mixtures *in vitro* appeared to substantiate this theory. It has since been recognized, however (Rosebury, 1944), that the lactobacilli used by Fosdick & Hansen (1936) were either peculiarly poor acid producers or were not adequately served by the media employed; and subsequent workers have shown that *Candida-Lactobacillus* mixtures are in fact less acidogenic than pure cultures of lactobacilli (Stephan & Hemmens, 1947) and likely therefore to reduce rather than enhance the acid factor of caries. But this is not the end of the story. Lilienthal (1950*b*) found a general increase in acid production when lactobacilli were grown in the presence of *Candida albicans*, and although his results show considerable variations we have also done similar tests the results of which supported his findings. The greatest increase in acid yield was obtained when heat-killed *Candida* cells were added to *Lactobacillus* cultures, indicating a nutritional rather than enzymic relationship between the organisms (see Young, Krasner & Yudkofsky, 1955). Yeasts and lactobacilli commonly occur together in nature and one such association was found by Challinor & Rose (1954) to depend upon at least thirteen nutritional inter-relationships. The heat and penicillin resistance of lactobacilli have also been shown to be increased by association with *Candida albicans* (Peppler & Frazier, 1942; Dreizen *et al.* 1956). Full explanations of these effects are not yet available, but acid dissimilation, enhancement of reducing conditions and proteolytic activity, and direct nutritional symbiosis have been suggested. Thus there does appear to be a correlation between the incidence of yeast-like fungi and lactobacilli in the mouth (Lilienthal, 1950*a*).

Other nutritional inter-relationships of a type which may occur in the mouth, for example between lactobacilli and streptococci, have been described (Nurmikko, 1952; Koft & Morrison, 1956), and this lends weight to the recent suggestion that there is a relationship between the incidence of *Streptococcus salivarius* and lactobacilli in the mouth (Cohen & Collins, 1958).

(b) *Antagonism*

The possibility that susceptibility to dental caries may be governed by antagonisms between various oral bacteria has stimulated research into such processes. Many reports are available, ranging from suggestions that soil bacteria producing antibiotics inhibitory to lactobacilli should be implanted in the mouth, to the demonstration of antagonisms between various members of the oral flora *in vitro* (Clough, Bibby & Berry, 1938; Hill *et al.* 1949). In some cases, as for example the recently described inhibition of oral lactobacilli by streptococci (Green & Dodd, 1956*b*), the effects do not appear to be due to acid or hydrogen peroxide, or nutritional competition, but to some undefined antibiotic factor. Whether this factor is in any way similar to the known antibiotic substances produced by lactic streptococci is not known.

(c) *Control*

Naturally, the search for practical ways in which the oral acidogenic bacteria, including lactobacilli, may be controlled or removed from the mouth, has been pressed forward vigorously. Among the agents tried, success has been claimed for penicillin, chlorophyll, vitamin K, ammonium salts and fluorine salts. The use of enzyme inhibitors to block the glycolysis cycle continues to be studied, and we are all familiar with the common use of surface active agents as components of tooth-pastes. Some of these suggestions may be useful, but others may involve such disturbance of the oral ecological system as to be quite dangerous and in view of the disastrous effects of certain other attempts by man to interfere in the balance of nature with inadequate background information of the subject, it will be wise to proceed with caution, and research.

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FACTORS AFFECTING THE IMPLANTATION OF LACTOBACILLI IN THE INTESTINE

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1. INTRODUCTION

THE THERAPEUTIC use of lactobacilli as an aid to the cure of a variety of gastro-intestinal disorders has been advocated for many years. But although some authors reported clear-cut results the literature on the subject reveals a certain amount of confusion. Early work was to some extent hampered by lack of knowledge about the members of the genus *Lactobacillus*, whose growth requirements were imperfectly understood. Also, the characteristics for differentiation of the various species had not then been accurately defined.

2. EARLY WORK WITH SOURED MILKS

Soured milks such as yoghurt, koumiss and kefir were used therapeutically in Europe and Asia long before the existence of bacteria was recognized. In the early days of bacteriology the use of soured milks was investigated and attempts were made to establish a scientific basis for their action; but many years elapsed before the problems associated with the implantation of lactobacilli in the intestine were fully understood. That this type of therapy may be successfully applied is only now becoming recognized.

Some of the first attempts to implant lactobacilli in the intestine and to treat various gastro-intestinal disorders were made with yoghurt and with cultures of *Lactobacillus bulgaricus*, the 'Bulgarian bacillus' as it was often called in those days. Milk soured with this organism was fed to subjects showing putrefactive changes in the intestine when on a normal mixed diet, and in all cases putrefaction decreased and the stools became normal with a predominantly Gram-positive flora, the undesirable bacteria being inhibited by the *L. bulgaricus* (Cohendy, 1906 *a, b*). The *Lactobacillus* appeared in the stools 8 days after the start of treatment and persisted for

at least a further 12 days. Similarly, when cultures of *L. bulgaricus* isolated from yoghurt were fed to mice there was a reduction in the total bacterial count of the faeces and a decrease in products of putrefaction (Belonovsky, 1907). The organism was established after 10 days' feeding and persisted for variable periods up to 2 months. Luerssen & Kuhn (1908), Herter & Kendall (1908) and Rahe (1915), however, considered that *L. bulgaricus* could not become established in the intestine and Rahe considered that though the organism did have a limited period of survival in the small intestine this was of little value to the host because that was not the site of the most active putrefaction. In 1910 Metchnikoff attributed senility and early death due to arterio-sclerosis to the toxic action of products of putrefaction in the intestine and expressed the belief that this could be overcome by the ingestion of large amounts of milk soured with *Bacillus* (*L.*) *bulgaricus*, suggesting that this organism, once established in the gut, would by transforming the intestinal flora reduce proteolysis and toxin production and thereby prolong life. Controversy over the question of the implantation of this organism raged for many years and the use of yoghurt for therapeutic purposes has even been mentioned in recent reports (Mayon-White, 1951; Wilcox, 1951), which have re-awakened interest in this matter.

Cheplin & Rettger (1922) were probably the first to give full details of methods used, cases treated and results obtained, and a very clear picture of their work is available. It is unfortunate that this was followed by the marketing of a wide variety of valueless lactobacillus preparations. These contained either mainly dead cell material or strains of lactobacilli that were foreign to the intestine and so could not become established. This resulted in negative results being obtained in several clinical trials and lactobacillus therapy came once more to be regarded as of dubious value. Extensive reviews of the early work on this subject were made by Frost & Hankinson (1931) and by Rettger *et al.* (1935).

3. THE CONDITIONS FOR SUCCESSFUL IMPLANTATION

Rettger *et al.* (1935) summarized the conditions on which the success of lactobacillus therapy depends, and their criteria are still valid. They showed that it is essential to use an intestinal strain, namely *L. acidophilus*, which now has clearly defined distinguishing characteristics (Rogosa *et al.* 1953; Wheeler, 1955; Sharpe, 1955), and also to administer a large number of viable cells. This, in the case of a liquid preparation, i.e. acidophilus milk, necessitates storage at a low temperature and for a minimum time. For a freeze dried preparation to contain a large number of living cells the method of drying, composition of the suspending medium and the manner of preparation are all important factors. With both types of preparation it is most important that the correct dose of viable cells be administered. It was recently found (Gordon, Macrae & Wheeler, 1957) that the most satisfactory dose was 10^8 – 10^9 viable lactobacilli daily. The administration of a daily total of 2.4×10^{10} viable cells caused a mild form of diarrhoea.

A further important factor in the successful implantation of these organisms is the diet. It is necessary to ensure either that patients undergoing treatment receive a diet with a high milk or lactose supplement, or that the important growth factors required by the lactobacilli are incorporated in the preparation.

4. THE BALANCE OF ORGANISMS IN THE INTESTINE

The main reason for implanting lactobacilli is to treat certain intestinal disorders in which the normal flora is either no longer present or failing to function in the usual manner. The delicate balance between the organisms constituting the flora, which is maintained by symbiosis and competition, can be upset in many ways. Antibiotics and other forms of treatment, for instance, may destroy one or more of the components of the normal flora (Garrod, 1955). Further, in various clinical conditions a particular group of organisms may show a large numerical increase or decrease (Cruickshank & Cruickshank, 1931). Diet and age also affect this balance, but to a much smaller extent.

It is now generally believed that lactobacilli constitute a large part of the intestinal flora in the healthy child or adult. Hermann & Kuhlman (1955) found that they constituted on the average some 50% of the normal flora, though diet could cause quite wide fluctuations. At one time *L. bifidus* was believed to be typical of the breast fed infant (Tissier, 1900) and that it was replaced by *L. acidophilus* when a mixed diet was introduced (Moro, 1905). More recently, however, this theory has been found to be only partly true. Gyllenberg & Carlberg (1958) demonstrated the predominance of a particular biochemical variety of *L. bifidus* in the stools of breast fed infants, but strains of *L. bifidus* have been shown to occur in large numbers in the adult (Mossel, 1959). Orla-Jensen, Orla-Jensen & Winther (1936) claimed that if milk was taken daily, *L. bifidus* would be present in large numbers in the intestinal canal of adults as well as infants.

Unlike the coli-aerogenes bacteria and the enterococci, which are also an essential part of the normal balanced flora, intestinal lactobacilli do not include any potential pathogens: on the contrary, they are usually regarded as beneficial. Metchnikoff (1915) stated that *L. bifidus* was absent in small children suffering from intestinal infections and that its reappearance was generally regarded as a good sign of recovery. Similarly, several of the workers who have studied various pathological conditions have attempted, as an important part of treatment, to produce an intestinal flora with a preponderance of lactobacilli (Ross & Dawes, 1954; Smythe, 1958).

5. STIMULATION OF THE NORMAL INTESTINAL LACTOBACILLI

Various methods have been used in attempts to stimulate the lactobacilli already present in the intestine. For example, the administration of lactose has long been advocated for both adults and infants (Rettger & Cheplin, 1921; Atkinson, Kratzer & Stewart, 1957). Generally, lactose was added in widely varying amounts to ordinary mixed diets, and there is evidence that this brought about increases in lactobacilli, decreases in putrefactive organisms and marked alleviation of constipation. In this country attempts have been made to treat gastro-enteritis in infants by adding lactose to the diet, e.g. by Ross & Dawes (1954) who administered up to 5% of lactose in the hope of producing an intestinal pH inhibitory to *Escherichia coli*, but found that the reduction of pH and increase in lactobacilli were only temporary. Similar work done by Zilberg (1956) also showed that the ensuing reduction in pH and increase in faecal lactobacilli were only temporary, even when 20% of lactose was

administered, and that the lactose appeared to exert no suppressive effect on the excretion of specific types of *E. coli*.

De Groot & Hoogendoorn (1957) found that feeding large doses of lactose to a variety of mammals caused a retardation of growth as well as loss of appetite and diarrhoea, and attributed these effects to lack of intestinal lactase activity. Thus, in view of the side effects and the temporary nature of the improvement, it appears that large doses of lactose are not entirely desirable for promoting a high content of lactobacilli. Raffle (1956) used yoghurt to supplement oxytetracycline in the treatment of infantile gastro-enteritis and found that in 19 out of 20 cases lactobacilli appeared in the stools and the pathogenic strains of *E. coli* disappeared. Yoghurt was used by Smythe (1958) in an attempt to establish a normal intestinal flora and to prevent staphylococcal enterocolitis in cases of kwashiorkor. Thirty-three patients received treatment, the effect of which was most marked in controlling the diarrhoea present in nearly all the cases. Yoghurt was also used with success in refractory cases of gastro-enteritis.

It would seem most likely that the benefits attributed to yoghurt are due partly to the inhibitory effects of the lactic acid (Seneca, Henderson & Collins, 1950; Emanuiloff & Natscheff, 1956), and partly to the stimulation of the normal intestinal lactobacilli (Orla-Jensen, Olsen & Geill, 1945). Distaso & Schiller (1914) observed that the faecal flora of patients fed with cell suspensions or milk cultures of *L. bulgaricus* consisted mainly of *L. bifidus*. Only small numbers of *L. bulgaricus* appeared.

Other attempts to produce an intestinal flora in which lactobacilli predominated have been described. β -Ethyl-N-acetyl-D-glucosamine has been added to milk in an effort to produce in artificially fed infants an intestinal flora similar to that of breast fed infants, and this substance does in fact promote a predominantly *L. bifidus* flora (Welch, 1956). The oral administration of either folic acid or soya bean flour was tried as a possible means of curing ammoniacal dermatitis in infants by promoting the growth of intestinal lactobacilli, and though the response to folic acid at the doses used was poor, the soya bean flour produced very good results in all cases (Miller, 1957).

Administration of all these preparations would therefore seem to be of some value in the restoration of the normal intestinal flora, but for their efficiency they are entirely dependent on there being sufficient viable lactobacilli already present in the intestine. It would seem more logical, particularly in view of these qualifying factors, to administer the actual intestinal lactobacilli, *L. acidophilus* or *L. bifidus*, in a medium capable of supporting their growth in the absence of other nutrients.

6. THE ADMINISTRATION OF *L. ACIDOPHILUS*

Smith (1924) found that when $2\frac{1}{2}$ pints of acidophilus milk were consumed each day the number of *E. coli* was substantially reduced and the flora of the stools consisted almost entirely of *L. acidophilus*. Constipation was relieved, but the results with chronic *Salmonella* carriers were disappointing. The feeding of lactose was less effective. Cheplin & Rettger (1922) treated chronic constipation and chronic

diarrhoea with a similar preparation, but at approximately half the dosage, and in some instances with the addition of lactose. They found that whereas some cases responded more quickly than others, a marked improvement was noted in every case in which the *L. acidophilus* content of the intestinal flora increased by 60–70%. Later, Rettger *et al.* (1935) treated patients suffering from a variety of diseases, including constipation, diarrhoea, mucous colitis, chronic ulcerative colitis and constipation accompanied by biliary symptoms, and in each of these groups 75–80% of the cases responded to treatment with *L. acidophilus*. Other workers (Portis & Albus, 1931; Stark *et al.* 1934) who used *L. acidophilus* for similar conditions have confirmed these findings.

In recent years the marked alteration in the intestinal flora brought about by the administration of antibiotics has aroused a new interest in *L. acidophilus* therapy, and there have been several reports of clinical work on this problem and on other conditions which might benefit by lactobacillus treatment (Gillespie *et al.* 1956).

Todd & Hopps (1955) encountered fatal cases of staphylococcal enteritis following antibiotic therapy and, with a view to limiting bowel sterilization, suggested the inclusion of antibiotic-resistant lactobacilli in the treatment. This was also suggested by Frazer (1958) when discussing the problem of antibiotic side effects. The effects of this type of therapy have been described by Gordon, Macrae & Wheeler (1957), who found that the co-administration of antibiotic-resistant strains of *L. acidophilus* with tetracycline prevented the rapid increase of staphylococci in the gut which occurred when the antibiotic was administered alone. This work was carried out with children between the ages of 1 and 13 years and, because its primary object was to study the effects of antibiotics and lactobacilli on the intestinal flora, only respiratory streptococcal infections and scarlet fever were included in the cases studied, enteric cases being omitted.

Various reports have been made about the clinical results obtained by using lactobacilli in conjunction with antibiotics. Gregor (1958, pers. comm.), using a preparation of *L. acidophilus* as a routine measure with all patients on long-term antibiotic treatment, found that the occurrence of vitamin deficiency and gastro-intestinal disorders was noticeably decreased. Manheim (1951) noted that acidophilus milk was of value in the treatment of anorectal complications, such as *pruritus ani* and fissures and abscesses *in ano*, which followed the oral administration of broad-spectrum antibiotics. Permanent cures were obtained with most cases and striking improvements with the remainder. Hoffmann (1959) treated a group of children suffering from dyspepsia and other intestinal disturbances with *L. acidophilus* in addition to antibiotics and found that 40% of the children in the control group receiving antibiotics but no lactobacilli developed moniliasis. Following the administration of *L. acidophilus*, however, these infected controls were cleared within 3–4 days and, provided that the administration of lactobacilli was continued until all clinical signs of dyspepsia had disappeared, the monilial infections did not reappear. These results confirmed earlier work by Marget (1957), who reported that a preparation of *L. acidophilus* was not only able to prevent the colonisation of the intestine by undesirable organisms but could also eliminate monilias which had already appeared as a result of antibiotic therapy.

An important finding in both these papers was that artificially fed healthy infants and also both breast and artificially fed dyspeptic infants showed striking weight gains when their diet was supplemented with *L. acidophilus*. These infants, whose weight increases before treatment were only 20–50 g/week, showed a remarkable improvement, the weight gains rapidly approximating to those of healthy breast fed infants. At the same time, healthy breast fed infants with a predominantly *L. bifidus* flora showed no similar weight gain with the *L. acidophilus* preparation. Experiments made by Robinson & Thompson (1952) on the effect of supplementing the diets of healthy infants with folic acid and *L. acidophilus* cultures, showed that during the first month of life bottle fed infants without these supplements did not gain weight as rapidly as breast fed infants. The inclusion of *L. acidophilus* in the diet, however, resulted in weight gains with bottle fed infants which approached those normally found with breast fed infants, though there was no effect on the weight gains of the latter group. Folic acid alone was without effect on either group.

Raaflaub (1957*a,b*) included a preparation of *L. acidophilus* as a routine in the diets of hospital maternity cases and found that this resulted in the elimination of staphylococcal and mycotic complications. It is interesting to note that these patients, even those with pyrexia, showed improvements in digestion, bowel activity and general condition. A lactobacillus isolated from healthy breast fed infants was also used for the treatment of dysentery in infants. With this it was found that 24–36 hr after administration of lyophilized organisms there was a pronounced clinical improvement, lactobacilli appearing in large numbers in the stools. It has been suggested that acute infective gastro-enteritis of children and infants should be treated with *L. acidophilus* in addition to antibiotics (Twining-McMath, 1959). This would mean that a stable protective intestinal flora was maintained while the pathogenic organisms were being eliminated. A preparation of *L. acidophilus* was also found to be of great value in reducing the incidence of offensive stools among geriatric patients who were being treated with certain laxatives (Jackson, 1958), especially those being fed with a nasal gastroduodenal tube. This was presumably due to the establishment in the intestine of a predominantly *Lactobacillus* flora, with the consequent elimination of putrefactive processes.

The role of *L. acidophilus* in the treatment of *Salmonella* and *Shigella* carriers has not been fully investigated. Portis & Albus (1931) treated a persistent carrier with acidophilus milk and found that coincident with the implantation of the lactobacilli the stools became free from typhoid organisms. Two cases treated by Flight (1959, pers. comm.) showed promising results, one remaining negative when examined 21 days after treatment had stopped, while the other, although still under treatment, was also negative at that stage. Smith (1924), however, did not find this type of therapy of value with chronic typhoid carriers.

The treatment of a wide variety of conditions with a preparation of *L. acidophilus* in tablet form was described by Winkelstein (1956). The conditions included in addition to some already mentioned, chronic constipation, functional diarrhoea, mucous and ulcerative colitis, sigmoid diverticulitis, megacolon and ileitis. Ulcerative colitis and ileitis did not respond to this treatment but favourable results were obtained with 90% of the other cases. In some instances relapses occurred after

treatment was stopped and a further period of administration was necessary before a completely satisfactory response was obtained.

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MICRO-ORGANISMS FROM CHICKEN MEAT RELATED TO BOTH LACTOBACILLI AND AEROBIC SPOREFORMERS

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SUMMARY: Certain Gram-positive, catalase negative rods with a homofermentative action on glucose, isolated from chicken meat, resembled lactobacilli in many respects but differed in their poor growth in media designed for lactobacilli and in other properties. The constituents of cell extracts and of the cell walls of these organisms showed some resemblance to those characteristic of the genus *Bacillus*, but no spores were found. Three groups were distinguished. Group 1 organisms were rod shaped or filamentous and non-motile, with no action on litmus milk, arginine or aesculin; those of group 2 were rod-shaped, non-motile, reduced litmus milk rapidly and hydrolyzed arginine and aesculin; and group 3 strains were short motile rods similar to those of group 2 in their effects on litmus milk, arginine and aesculin.

THE ORGANISMS described here were Gram-positive catalase negative rods isolated from chicken meat during irradiation experiments. In one experiment the meat was minced and stored in nitrogen at 5° (Thornley, 1957) while in the other whole, eviscerated carcasses were stored at 3° in air in polythene bags. Although not detected initially, during storage the organisms concerned became numerous on both control and irradiated samples and in some cases reached 10⁸/g. Samples were plated on various media, and most of the organisms were isolated from digest, yeast extract and blood plates; a few were obtained from tetrazolium agar (Barnes, 1956) but only one grew on the acetate agar of Mabbitt & Zielinska (1956) which was used as a selective medium for lactobacilli. It was uncertain, therefore, whether the organisms were in fact lactobacilli, and accordingly 59 strains were studied in detail.

METHODS

In most cases two methods of testing were applied, namely tests appropriate for lactobacilli, with incubation at 30°, and more general methods at 25°.

Media

Yeast glucose broth. The yeast glucose broth described by Sharpe (1955) as medium A was used as a basic medium; and for tests of ability to grow at different pH levels this medium was used with the pH adjusted. For some of the culturing Hartley's digest agar was used, prepared according to Mackie & McCartney (1948), and also digest broth and agar containing 0.3% of yeast extract.

MRS medium. This medium, in which some of the organisms were grown, was specially designed for lactobacilli (de Man, Rogosa & Sharpe, pers. comm.) and the formula will be published later.

Medium for inducing the formation of spores. The medium described by Charney, Fisher & Hegarty (1951) was used.

Physiological tests

Litmus milk. Tests were made using plain litmus milk, as described by Mackie & McCartney (1948), and also in yeast (0.3%) glucose (1%) litmus milk.

Aesculin hydrolysis. This was tested by the method of Naylor & Sharpe (1958).

Arginine breakdown. Briggs' medium (Briggs, 1953) was used in the test for the production of ammonia from arginine, and Thornley's medium (Thornley, 1960) to indicate an alkaline pH shift in the presence of arginine. With some strains Thornley's medium proved inadequate for growth and was modified by the addition of 0.3% of yeast extract.

Fermentation reactions. These were done in peptone water containing 1% of the carbohydrate, and also in the basal sugar medium used for lactobacilli by Wheeler (1955) with 0.2% of carbohydrate.

Heat survival. These tests were done according to the method of Briggs (1953), except that the medium was yeast glucose broth and the inoculum consisted of 0.03 ml of a 24 hr culture in yeast glucose broth. With a few strains, tests were also done with a suspension containing a comparable number of cells obtained from slopes of the sporulation medium which had been incubated for 7 days at 25°.

Nitrate reduction. The medium contained (as % w/v): Evans peptone, 1.0; Lab-Lemco, 1.0; NaCl, 0.5; glucose, 0.5; Yeastrel, 0.3 and KNO₃, 0.1; the final pH was 7.0.

Gas from glucose. The method of Gibson & Abd-el-Malek (1945) was used.

RESULTS

General properties of the organisms

The organisms were all Gram-positive rods, with some variations in the rod shape in one group. When cultures on the special sporulation medium were examined no spores were seen in Gram-stained smears. Catalase tests on cells grown in yeast glucose broth, yeast glucose agar, yeast extract broth and digest agar, i.e. media with and without added carbohydrates, were generally negative but in some cases the results were very weakly positive.

All the cultures grew at 15° and not at 45°. In accordance with their low growth temperature, their heat resistance was also low, as none of the cultures survived 30 min at 60°, whether they had been grown previously for 1 week on the sporulation medium or overnight in yeast glucose broth. This provided further evidence against the formation of spores.

All the strains were tested for growth on acetate agar, but only two grew; these were originally isolated from tetrazolium and acetate agar and resembled typical lactobacilli much more closely than did the other strains. They were homo-fermentative low temperature strains, but they did not correspond to either *Lacto-*

bacillus casei or *L. plantarum* in their fermentation reactions, and they will not be considered further. The remaining organisms varied in their ability to grow at low pH levels (see later) and as some were able to initiate growth in broth at pH 4.5 it seems unlikely that their inhibition on acetate agar was due to its pH of 5.35.

Unlike the lactobacilli, all the strains grew as well, if not better, on agar plates incubated aerobically than on plates incubated anaerobically. In the MRS medium for lactobacilli there was poor, slow growth. Nitrate was not reduced by any of the organisms. No gas was produced from glucose, and when the fermentation products from glucose of one strain in each group were analyzed, they were found to consist of lactic acid with traces of acetic acid. The organisms therefore carried out a typical homolactic fermentation. In addition to glucose, all the strains fermented amygdalin, cellobiose, fructose, maltose, mannose, salicin, sucrose and trehalose. Only two strains fermented melibiose, and none fermented arabinose, rhamnose, sorbose or xylose.

Differentiation of groups

The organisms were divided into three groups based on the further differentiating characters, both morphological and biochemical, shown in Table 1.

Table 1. *The characteristics of three groups of organisms isolated from chicken meat*

Character	Characteristics* of group		
	1 (14 strains)	2 (37 strains)	3 (8 strains)
Morphology	Rods or filaments	Rods	Short, thick rods
Motility	—	—	+
Change in yeast glucose litmus milk	—	R ¹ AC	R ¹
NH ₃ from arginine†	—	+	+
Aesculin hydrolysis	—	+	+
Fermentation of:			
Galactose	—	+(17), -(20)	+
Lactose	— or slow +	+(19), -(18)	— or slow +
Melezitose	—	+(26), -(11)	—
Raffinose	—	+(10), -(27)	—
Dextrin	— or slow +	+(37)	— or slow +
Inulin	—	+(11), -(26)	+(7), -(1)
Glycerol	+	+(30), -(7)	—
Mannitol	+	+(13), -(24)	—
Inositol	+	+(7), -(30)	—
Final pH in yeast glucose broth	4.6–4.8	4.1–4.5	4.8–5.1

* +, present; —, absent. R¹, reduced in 1 day or less; A, acid; C, clot. Bracketed figures are the numbers of strains giving the reaction indicated.

† Medium of Thornley (1960), with 0.3% of yeast extract included for tests on organisms of group 3.

Group 1. Fourteen strains were placed in this group. They were all from whole eviscerated chicken which had been irradiated and stored at 3°, and were distinguished from the organisms of the other groups mainly on morphological criteria.

On solid medium, whether incubated aerobically or anaerobically with CO₂, the strains formed rods of medium length, but in liquid medium chains, filaments and spiral or swollen forms appeared. This was rather more marked when the presence of carbohydrates led to acid conditions in the medium, but it also occurred in yeast extract broth at a pH between 7 and 8; filament formation seemed, however, to depend on temperature. Plate 1 shows an organism of group 1 grown in glucose peptone broth at 30° (a), 25° (b) and 15° (c). Although growth was considerably slower at 15°, the short rods which developed at that temperature would usually be regarded as the 'normal' form of the organisms. The spiral forms sometimes seen after 24 hours' growth at 25° are illustrated in Plate 1 (d). This, like the other photographs, was taken by phase contrast, and the cells were forced into one plane by allowing the surrounding liquid to dry until the coverslip almost touched the slide. However, the marked curvature of some cells, which form 'C' and 'S' shapes, can be clearly seen.

Most organisms in this group were initially isolated on aerobic plates, and when their growth was later studied under different conditions they gave larger colonies after aerobic growth than when incubated in hydrogen plus 10% CO₂. The colonies varied in morphology, a single culture frequently giving rise to more and less translucent types on the same plate. Other characters are listed in Table 1. This group differed from the other two in the absence of any reaction with litmus milk, arginine and aesculin. As a rule only the sugars already mentioned were fermented, and acid was produced from the three alcohols glycerol, mannitol and inositol. The final pH values in yeast glucose broth ranged from 4.6–4.8, and growth was initiated in broth at pH values of 5.5 and 9.0, but not usually at pH 5.0.

The peculiar morphological variations together with the marked preference for aerobic conditions were sufficient to distinguish this group from typical lactobacilli and Rogosa (1959: pers. comm.) suggested that they might be related to *Leptotrichia*. From the published descriptions of *Leptotrichia* they seemed most similar to the organisms described by Bøe & Thjøtta (1944) and by Hamilton & Zahler (1957): these were long rods in pairs, which sometimes gave rise to spiral and swollen forms in old cultures; they were homofermentative lactic acid producers, with a final pH in yeast glucose broth of 4.6–4.8, and gave a very similar pattern of fermentation of sugars. However, these organisms differed markedly from ours in being Gram-positive only in very young cultures (6 hr) and in being strongly anaerobic on first isolation. The aerobic *Leptotrichia dentium* of Baird-Parker & Davis (1958) differed from our organisms in being consistently filamentous, with frequent branching.

Group 2. Organisms of this group were most frequently encountered. Many strains were isolated from minced chicken meat after anaerobic spoilage, and came from both control and irradiated samples. Such strains were described as of group A by Thornley (1957), and 37 strains from this source have been studied. Organisms with similar characteristics have also been isolated from other sources. Beef, minced and packed anaerobically, was stored for 3 weeks at 5° and then sampled. Gram-positive catalase negative rods were predominant in all samples in numbers of about 10⁸/g and, of 48 isolates, 20 proved to belong to group 2. In addition, an organism isolated from contaminated blood plasma by Miss C. Shaw of the Lister Institute of Preventive Medicine belonged to this group.

In their morphology on different media, these organisms varied from medium length to coccoid rods (Plate 1 (e)). A tendency for pairs of cells to remain joined at an angle is reminiscent of coryneforms, but this appearance has also been seen among typical lactobacilli, e.g. *Streptobacterium plantarum* no. 5 of Orla-Jensen (1919), Plate XLIII.

The characteristics of organisms of this group are shown in Table 1. Rapid reduction of litmus milk, whether alone or with added yeast extract and glucose, was a striking feature. With typical lactobacilli, acid formation and clotting occur before reduction. The test for the production of ammonia from arginine was done by Briggs' method and most strains gave positive results, but with Thornley's medium, designed to test *Pseudomonas* strains, all members of this group gave positive results. The occasional differences may have been due to the high concentration of glucose (2%) in Briggs' medium, as this has been shown by Niven, Smiley & Sherman (1942) to influence the results of the arginine test. With the homofermentative lactobacilli, a positive reaction by Briggs' method is given by some strains only of *L. leichmannii* and *L. delbrueckii*, two of the high temperature group which differ from our organisms in many respects. Aesculin was hydrolyzed by all strains in group 2.

Table 2. *The carbohydrate fermentation patterns among organisms of group 2*

Carbohydrate	Fermentation* pattern									
	A	B	C	D	E	F	G	H	I	J
Galactose	—	+	+	—	—	—	—	+	—	+
Lactose	—	+	+	—	—	—	+	+	+	+
Melezitose	+	+	—	+	+	+	—	+	+	+
Raffinose	—	+	—	—	+	—	—	—	—	+
Dextrin	+	+	+	+	+	+	+	+	+	+
Inulin	—	+	—	—	—	+	+	—	+	—
Glycerol	+	+	+	—	—	+	+	+	+	—
Mannitol	—	+	—	—	—	+	+	+	+	+
Inositol	—	—	—	—	—	+	+	—	+	—

* +, acid; —, no change.

Table 3. *The relation between source and fermentation pattern among organisms of group 2*

Source of strain	Nos. of strains with fermentation pattern*									
	A	B	C	D	E	F	G	H	I	J
Chicken	2 (2)	6 (5)	4 (3)	0	0	2 (2)	0	0	1 (1)	1 (1)
Beef	8 (4)	0	4 (2)	4 (2)	2 (2)	0	1 (1)	1 (1)	0	0
Blood plasma	0	1†	0	0	0	0	0	0	0	0

* See Table 2. The numbers of samples positive are in brackets.

† A contaminant.

Fermentative ability varied considerably, as shown in Table 1. Ten different combinations of fermentation reactions were actually observed, as shown in Table 2. The commonest were A, B, and C, all of which were shown by strains from two

different sources (Table 3). The final pH values in yeast glucose broth were between 4.1 and 4.5, and most strains could initiate growth in media at pH 4.5 though not at 4.0. This acid tolerance seemed comparable with that of typical lactobacilli.

Group 3. These organisms were isolated from minced chicken meat which had been irradiated and stored anaerobically at 5° for 8 months; they constituted group C of Thornley (1957). Eight strains were studied in the present work. They differed from those in group 2 in being shorter, thicker rods, and in being motile. Plate 1 (f) shows an electron micrograph of a typical example. A pair of cells is shown and, although only three flagella are attached, it appears that the arrangement is peritrichous.

Ammonia was produced from arginine by all the strains, when Briggs' method was used. Thornley's medium gave negative results for some strains, probably due to inadequate growth, since this medium with 0.3% of yeast extract added gave positive results for all the strains. The organisms also hydrolyzed aesculin and rapidly reduced litmus milk, as did those of group 2. The final pH value in yeast glucose broth was higher than that with group 2 organisms, being between 4.8 and 5.1. The range of fermentation reactions carried out by this group was much more restricted than with group 2 strains (Table 1). In addition to the carbohydrates fermented by all groups, galactose was fermented by all group 3 strains, inulin by all but one, and there was slow acid formation from lactose and dextrin with some strains.

Cell constituents of the three groups

Cell extracts of representative strains of each group were studied chromatographically by the method of Mattick *et al.* (1956), to reveal the amino acids and polypeptides present. Cultures of *L. plantarum*, *L. casei*, three corynebacteria and *Bacillus*

Table 4. *The relation between the cell wall composition of Corynebacterium and Lactobacillus species (after Cummins & Harris, 1956) and that of unknown strains*

Substance found	Presence or absence* in cell walls of					
	<i>Corynebacterium</i> (8 spp.)	<i>Lactobacillus</i> (6 spp.)	<i>L. plantarum</i> (4 strains)	Unknown strains, group		
				1	2	3
Arabinose	+	— or **	— or **	—	—	—
Rhamnose	+ or —	— or +	—	—	+	—
Galactose	+	— or +	— or +	—	+	+
Glucose	+ or —	** or +	+	—	—	—
Mannose	+ or **	— or +	— or +	—	—	—
Glucosamine	+	+	+	+	+	+
Galactosamine	+ or —	— or +	—	—	—	+
Muramic acid	+	+	+	+	+	+
Aspartic acid	†	+	†	**	**	**
Alanine	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+
Lysine	†	+	†	**	**	**
Diaminopimelic acid	+	†	+	+	+	+

* +, present; —, absent; **, absent, or present only in traces; †, not recorded.

polymyxa were used as controls. All our organisms showed characteristics quite distinct from those of the corynebacteria, but had certain features in common with some heterofermentative lactobacilli and with some species of *Bacillus* previously examined by Jayne-Williams & Cheeseman (1960).

Cell wall constituents were also investigated; Cummins & Harris (1956) had found that the amino acids of cell wall extracts of various genera of Gram-positive bacteria had characteristic patterns, and their results for *Corynebacterium* and *Lactobacillus* species are given in Table 4. The *Corynebacterium* species contained diaminopimelic acid (DAP) but lacked aspartic acid and lysine, and were also characterized by the presence of arabinose among the sugars. Most of the lactobacilli studied (6 species) contained aspartic acid and lysine, with no DAP, whereas *L. plantarum* strains had the same amino acids as *Corynebacterium*. So also had most of the *Bacillus* species tested, although one, *B. sphaericus*, resembled a typical lactobacillus. As shown in Table 4, one strain from each of our three groups was tested, and all contained DAP with no aspartic acid or lysine, and they all lacked arabinose. They therefore resembled *L. plantarum* (the only lactobacillus species known to contain DAP) and most *Bacillus* species in cell wall constituents.

DISCUSSION

The organisms described resemble *Bacillus* spp. in the constituents of both cell walls and cell extracts; however, no spores could be detected and they were catalase negative. They showed some morphological resemblance to *Corynebacterium*, especially the strains of group 2, but the cell wall and cell extract constituents differed markedly from those of corynebacteria and, moreover, the organisms, as well as being catalase negative, failed to reduce nitrate. Their nearest relationship therefore appears to be to the lactobacilli of the low temperature homofermentative group. They differ from these in being relatively easy to grow on ordinary media, including media lacking in carbohydrates, in giving poor growth in the special MRS medium for lactobacilli and in failing to grow on acetate agar. Strains of group 1 show differences in morphology from lactobacilli, with some resemblance to *Leptotrichia*, while those of groups 2 and 3 are unusual in the hydrolysis of arginine and the rapid reduction of litmus milk. The motile organisms of group 3 do not seem to correspond to any of the motile lactobacilli described hitherto (Harrison & Hansen, 1950; Hays & Riester, 1952; Mann & Oxford, 1954; Deibel & Niven, 1958).

Our organisms were isolated from meat held under unusual storage conditions. The combination of low temperature and anaerobic conditions is not normally used in practice with fresh meat and consequently the spoilage flora corresponding to these conditions has not previously been studied. Our results with beef stored in this way suggest that organisms similar to those described are common in these conditions. It therefore seems premature to give these organisms a definite place in any system of classification until more strains of a similar nature have been isolated and investigated; this should throw more light on the natural relationships of the group.

This work has been carried out as part of the programme of one of us (M.J.T.) at the Low Temperature Research Station, Cambridge.

We should like to thank Dr. C. S. Cummins of the London Hospital for his work on the cell wall constituents, Dr. G. C. Cheeseman of the National Institute for Research in Dairying for the chromatographic study of the cell extracts, and Mr. R. W. Horne of the Cavendish Laboratory, University of Cambridge for the electron micrograph.

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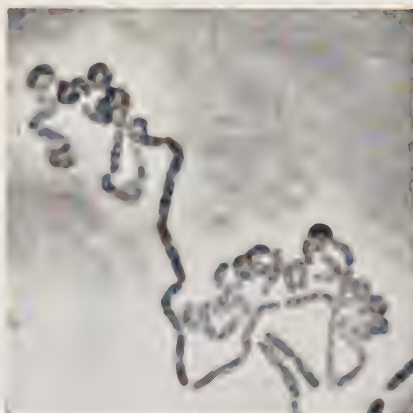
EXPLANATION OF PLATE

- (a) An organism of group 1, grown for 24 hr in yeast glucose broth at 30°. Phase contrast, $\times 700$.
- (b) As (a), grown at 25°. Phase contrast, $\times 700$.
- (c) As (a), grown for 2 days at 15°. Phase contrast, $\times 1700$.
- (d) As (a), grown for 24 hr at 25°. Phase contrast, $\times 1700$.
- (e) An organism of group 2, grown for 24 hr in yeast glucose broth at 25°. Phase contrast, $\times 1700$.
- (f) An organism of group 3, grown in yeast extract broth for 24 hr at 25°. Electron micrograph, gold-shadowed preparation, $\times 12000$.

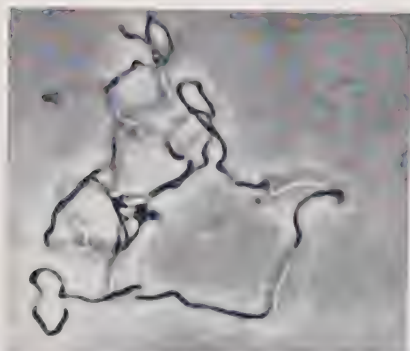
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(a)



(d)



(b)



(e)



(c)



(f)

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(Symposium on The Lactobacilli: Paper VI)

SOME SPECIAL CHARACTERISTICS OF THE CIDER LACTOBACILLI

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1. INTRODUCTION

CIDER LACTOBACILLI differ from other lactobacilli because of the unusual conditions under which they grow. Since their environment has a marked influence on their behaviour, a short description of apple juice is therefore given.

All apple juices contain fructose, glucose, sucrose and traces of xylose. Fructose occurs in concentrations of 6-10% while glucose and sucrose are less abundant, usually being present at concentrations of 1-2%. Sucrose is rapidly inverted when fermentation starts. A further sugar, arabinose, may sometimes be detected in minute amounts at the end of fermentation. Nitrogenous matter occurs in concentrations of 5-15 mg/100 ml and contains 12-14 amino acids of which the most abundant are aspartic acid, asparagine and glutamic acid (Burroughs, 1957). There are also mineral salts, B-group vitamins, tannins and organic acids (Phillips, Pollard & Whiting, 1956). The most abundant acids are malic, quinic, citramalic, chlorogenic, coumaryl-quinic, shikimic and citric.

It is thus clear that apple juice would be a suitable growth medium for many bacteria if it were not for its low pH of 3.0-4.6 and the fact that the environment is constantly being changed in composition by the activities of yeasts. During fermentation the sugars, with the exception of xylose, are fermented to ethanol giving concentrations up to 6% (v/v) and sometimes higher if sugar has been added.

As the fermentation proceeds the conditions become more anaerobic due to the uptake of oxygen and evolution of carbon dioxide by the yeasts. Much of the nitrogenous material is removed initially by the yeast cells but some is later replaced at the end of the fermentation when they are autolyzing (Burroughs & Carr, 1957).

Thus the lactic acid bacteria which occur in ciders must be able to withstand conditions of low pH and increasing ethanol concentration. They must also be able to survive a period of nitrogen starvation which occurs as a consequence of intensive yeast growth. There is however tentative evidence from experiments conducted jointly with Dr. G. C. Whiting (unpublished work), that 3-methyl- and 2-methyl-2,3-dihydroxybutyric acids, thought to be excreted by the yeasts (Whiting, 1959), can be used by certain lactic acid bacteria instead of valine, an amino acid which is essential for all types of cider lactic acid bacteria (Carr, 1956). For the most part the yeasts and bacteria are in competition for nutrients such as carbohydrates, and since the bacteria grow more slowly they must also be able to utilize substances not metabolized by the yeast. Under these conditions it is not surprising that certain specialized organisms have been selected.

2. TYPES OF ORGANISMS

The lactic acid bacteria of ciders are limited to three groups, namely, hetero- and homofermentative rods and heterofermentative cocci. Of these, the heterofermentative rods are mainly related to the species *Lactobacillus pastorianus* or *L. brevis* while the cocci resemble an organism often described in the Continental wine literature as *Bacterium gracile* which is, in the author's opinion, a non-slime-forming *Leuconostoc*. The heterofermentative species are more common than the homofermentative species, which occur only sporadically. Organisms belonging to the genera *Streptococcus* and *Pediococcus* have never been isolated. Most ciders contain lactic acid bacteria at some time, and such organisms are particularly abundant in low-acid ciders (i.e. with an acidity of less than 0.3% expressed as malic acid).

3. THE EFFECT OF pH ON THE GROWTH AND METABOLISM OF CIDER LACTOBACILLI

The most important characteristic of cider lactobacilli, imposed by their normal habitat, is their tolerance of very acid conditions. Table 1 illustrates this. Individual strains differ to some extent and the values given are averages. Both groups of heterofermentative organisms have the same optimum pH range and both are able to grow at pH values encountered in the most acid ciders. In contrast the homofermentative rods appear to be less well adapted to growth in highly acid conditions, since their optimum range is considerably higher than that of the other two groups and the minimum pH at which growth occurs would enable them to grow only in ciders of low acidity.

The fact that these organisms carry out their normal activities under conditions of low pH is of importance in influencing the choice of pH for test media used in their classification. Vaughan, Douglas & Fornachon (1949) recognized this fact when working with a wine bacterium, *L. hilgardii*. They showed that this organism could

ferment only xylose and fructose at pH 6.8–7.0, while at pH 4.5–5.5 glucose, galactose, maltose, lactose, sucrose and raffinose were also fermented.

Table 1. The optimum, minimum and maximum pH values for growth of the main groups of lactic acid bacteria of cider

Group	Optimum pH range	Minimum pH	Maximum pH
Heterofermentative rods	4.0–6.0	3.2	7.2
Homofermentative rods	5.8–7.0	4.0	7.4
Heterofermentative cocci	4.0–6.0	3.2	6.5

A similar phenomenon occurred with a heterofermentative rod which has been called *Lactobacillus pastorianus* but which might equally well have been called *L. brevis*. This organism was tested for its ability to utilize malic acid over the pH range 3.6–5.2 in steps of 0.2 and the acids produced were examined chromatographically. Although the usual end-products for the dissimilation of malic acid are lactic acid and carbon dioxide, heterofermentative bacteria invariably produce traces of succinic acid, and this organism was no exception. As Fig. 1 shows, at pH 3.6 the lactic acid

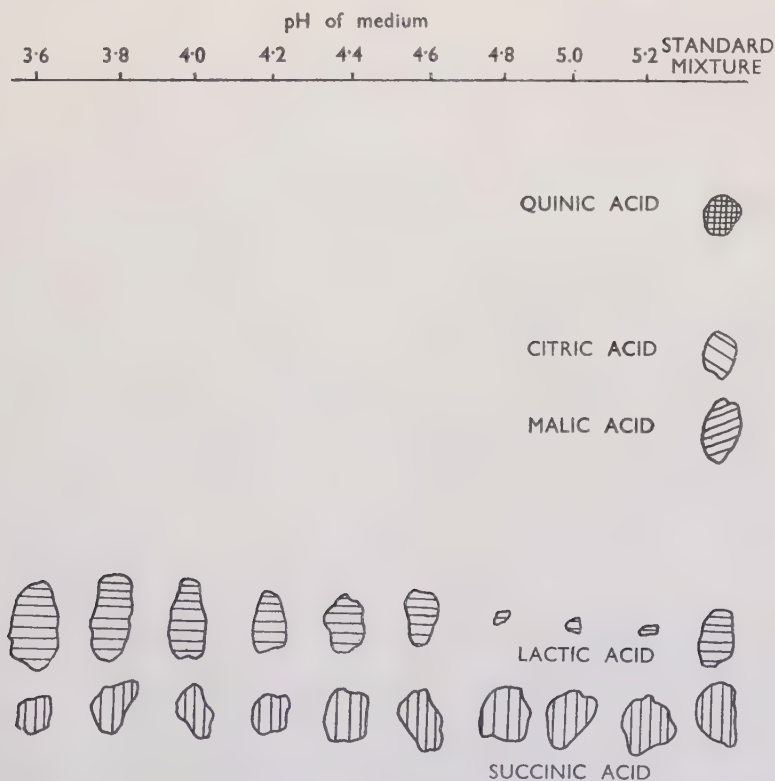


Fig. 1. A chromatogram tracing, showing the effect of the pH of the medium on the end-products of malate fermentation by a cider *Lactobacillus* sp.

exceeded the succinic acid; but with increasing pH the lactic acid gradually diminished until at pH 4.8 production of this acid had almost ceased. Conversely, the succinic acid had increased. Thus at the extremes of the pH range used, the results were very different. Although this test is not used for classifying these organisms, it illustrates the effect of pH upon acid metabolism and emphasizes the importance of pH in such studies. Similarly the usual sugar fermentation tests carried out at neutrality give only limited information about these organisms. Under conditions as acid as the normal habitat a wider range of sugars is fermented, and this has obvious implications of importance in taxonomic studies.

4. SUGAR FERMENTATIONS

Perhaps the most widely used test for the classification of lactobacilli has been the fermentation of various carbohydrates. All isolates from cider were tested against an extensive range of sugars and related compounds at pH 4.8. Under these conditions both groups of heterofermentative organisms showed the typical preference for arabinose, this sugar often having been fermented before any sign of activity could be observed in any of the other sugars. The next most readily metabolized sugar was fructose, which was invariably fermented by these organisms in preference to glucose. Indeed, one heterofermentative rod related to *L. brevis* was unable to utilize glucose at all while fructose was vigorously metabolized. In addition to acid, the metabolism of fructose always gave rise to mannitol. The detection of this hexitol by paper chromatography was used as a confirmatory test for heterofermentation.

Only a few cocci fermented lactose and this also applied to the homo- and heterofermentative rods. This is surprising, as nearly all the lactobacilli listed in the 7th edition of Bergey's Manual (Breed, Murray & Smith, 1957) are able to ferment lactose. The inability of the cider organisms to ferment this sugar indicates a lack of mechanism for splitting the β -linkage between the component hexoses. In contrast, nearly all the organisms fermented maltose, thus indicating the presence of an α -glucosidase which was coupled with their ability to ferment α -methyl-glucoside. Thus, the pattern of sugar fermentations of all the organisms allows them to grow well in sweet ciders since, without exception, all could ferment the most abundant sugar, fructose. As many of the organisms could ferment xylose and arabinose, survival after yeast fermentation would be possible for short periods even if no alternative substrates were available.

5. SLIME PRODUCTION

The lactic acid bacteria best known for slime production are two of the three species within the genus *Leuconostoc* which are able to produce dextran from sucrose. This property is unstable and should not, in the author's opinion, be used as a specific characteristic. Although many strains of heterofermentative cocci which resemble *Leuconostoc mesenteroides* in most properties except slime production have been isolated from cider, only a single strain has ever been encountered which produced a slimy polysaccharide. This was a coccus described by Millis (1951), which grew poorly except in apple juice medium and lost its slime forming ability so readily

that it was not possible to identify the substance from which the slime was synthesized. In this connection Pederson & Albury (1955) have shown that by transferring certain non-slime-forming, sucrose-fermenting leuconostocs in media of vegetable origin (grape juice, tomato juice, etc.) it was possible to induce dextran production. However, none of the potential slime-forming cocci from cider, even when subcultured in apple juice for long periods, has shown signs of producing slime.

There is, however, one troublesome slime-forming heterofermentative rod related to *L. brevis* which causes 'ropy' cider. It was first isolated and described by Millis (1951). It can build slime from glucose, fructose and maltose but not from sucrose, which it does not even ferment. Slime produced by this organism has been examined by Barker, Bourne, Salt & Stacey (1958) who showed that the products of hydrolysis contain glucose, galactose, arabinose and mannose: this would suggest that it is much more complex than dextran. References to slime-forming heterofermentative *Lactobacillus* species are rare, and the only other organism which appears to be at all similar to the cider isolate is one mentioned by Shimwell (1949) called *L. pastorianus* var. *brownii*, which unfortunately lost its slime-forming ability before he could identify the substance from which it was formed. The cider organism is, however, a more persistent slime-former and can cause considerable economic loss if its activities are not detected early.

6. ORGANIC ACID METABOLISM

It has already been shown that the lactic acid bacteria of ciders are able to metabolize a fairly wide range of sugars including those which occur abundantly in apple juice. But these bacteria are in competition for such substances with the yeasts, which grow more rapidly and thus largely deprive them of sugars. If a lactic acid bacterium is to survive under such conditions it must have the ability to utilize carbon sources which are not metabolized by the yeasts. Thus, in the absence of sugars, organic acids which are relatively unaffected by the yeasts are utilized by the bacteria.

Malic acid. This occurs in the greatest quantity and most cider bacteria, both homo- and heterofermentative types, are able to metabolize this acid. Hence lactic and some succinic acid occur in dry ciders as frequently as malic acid.

Citric acid. Although this acid occurs in very small amounts in apple juices it is present in much higher concentrations in pear juices. The metabolism of this acid is carried out mainly by heterofermentative rods and cocci which produce acetic and lactic acids. In contrast, the number of homofermentative organisms able to utilize this acid is very small.

Lactic acid. This acid occurs frequently and in large quantities in ciders but only in traces in apple juices. Its widespread occurrence in ciders is a direct consequence of the activity of lactic acid bacteria. None of the organisms isolated was able to utilize lactate either in ciders or under test conditions. Indeed, it would be a very unusual lactic acid bacterium that was able to utilize its own end-products; yet it is stated in the 6th and 7th editions of Bergey's Manual (Breed, Murray & Hitchens, 1948; Breed, Murray & Smith, 1957) that *L. brevis* is able to utilize calcium lactate, a statement which from work in this laboratory is open to doubt.

organisms are able to ferment than tests with neutral media. Also, competition between the fast growing yeasts in cider and the bacteria which grow more slowly has led to the selection of bacterial strains able to metabolize substances (organic acids) not utilized by related bacteria from other habitats. Even the type of polysaccharide produced by the only slime-forming rod isolated differs from the usual dextran from sucrose pattern, common to most slime-forming lactic acid bacteria. This would appear to be related to the fact that sucrose has only a temporary existence in a fermenting cider.

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(Symposium on The Lactobacilli: Paper VII)

CONTRIBUTIONS TO KNOWLEDGE OF THE MALO-LACTIC FERMENTATION IN WINES AND CIDERS. II. THE GROWTH PROMOTING EFFECT OF YEAST EXTRACT ON LACTIC ACID BACTERIA CAUSING MALOLACTIC FERMENTATION IN WINES

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SUMMARY: It has been shown that yeast extract and proteose peptone promote the growth of the species of *Lactobacillus* and *Leuconostoc* responsible for the malolactic fermentation in wines. On treatment of the yeast extract with 96% ethanol the growth promoting components remained primarily in the alcohol soluble fraction. Ion exchange experiments showed that the active substances were probably simple nitrogen compounds (possibly streptogenine-active substances) which progressively lost their activity with increasing acid hydrolysis. The active substances were concentrated by paper chromatography.

THE MALOLACTIC fermentation is of great importance in the climatically less favoured zones which are marginal for the cultivation of grapes, because fresh wines from those zones have so high an acid content that they are too unpalatable to be drunk without further development.

The total acid content of wine consists of tartaric and malic acids in roughly equal proportions. During the period of development this acid content must be reduced from an average of 7-10 g/l. to 4-5 g/l. In most countries it is permissible to effect partial de-acidification artificially with calcium carbonate, but this in itself is insufficient to produce wines of high quality. The retention of malic acid in the wine causes it to have an unripe flavour and character; hence a palatable product cannot be so obtained. Recently, other attempts have been made to produce such wines artificially by stopping fermentation while sugar remains; but these products too are rejected by wine lovers.

It is for this reason that even today the malolactic fermentation is as important as the alcoholic fermentation in the development of quality products in many wine regions. The bacterial conversion of malic acid into lactic acid and carbon dioxide results in a decrease of the total acid of the wine and the simultaneous ripening and harmonization of the taste. Unfortunately, these important processes in the development of a wine take place less regularly and certainly than the alcoholic fermentation, and they are sometimes delayed for months despite attempts to stimulate them. It is therefore understandable that wine producers are greatly interested in the artificial induction and control of this process, similar to that used in alcoholic fermentation.

The biological decomposition of acid, and the bacteria responsible, were first thoroughly described early in this century in the classical work of Müller-Thurgau & Osterwalder (1913), and their work was followed by numerous studies on the same topic by other authors: but knowledge of the systematic classification of the malolactic bacteria is now much improved. In wines there are homofermentative species which appear closely related to *Lactobacillus plantarum*; and in addition, heterofermentative species, the predominant forms of which have been identified as *L. buchneri*, *L. brevis*, *L. fermenti*, *L. trichoides* and also species of *Leuconostoc*. Further, we know the growth factor requirements of a few important strains and can cultivate them in completely synthetic media. Unfortunately, however, it is still impossible to induce and to control this important process with any certainty in practice.

Even the earliest authors (e.g. Koch, 1900) noted that development of the malolactic fermentation in wines must be related to the yeasts. It was often observed that resting yeast cells beneficially influenced the development of the malolactic bacteria; and for centuries, in practice, this development was stimulated by adding yeasts from another wine. These often effective measures, in the light of our work, cannot be explained solely on the basis that they gave rise to heavier inoculation of the wine by malolactic bacteria. From earlier studies (Lüthi, 1953; Hochstrasser, 1955; Lüthi, 1957) it was plain that the prior or simultaneous development of certain yeasts, bacteria or moulds greatly influenced particular slime-forming streptococci in their adaptation to low pH values and in the intensity of their consequent development. Our observations now indicate that this is also true for some of the lactic acid bacteria responsible for the malolactic fermentation in wines.

We have studied several strains of malolactic bacteria which we isolated from wine, particularly as regards their requirements in the medium. Their systematic classification has not yet been completed: they are heterofermentative and seem closely related to *Leuconostoc mesenteroides* and *Leuc. citrovorum*. Our work was chiefly concerned with the growth stimulating effect of yeast extract, and it is noteworthy that this effect is very similar to that of proteose peptone.

MATERIALS AND METHODS

Organism. A single strain of malolactic bacteria designated Pf 1 was studied.

Growth. This was estimated by measurement of turbidity in an EEL nephelometer.

Medium. The basal medium was a modification of Bacto folic acid assay medium B 318 (Difco Manual, 1953), which contains the necessary growth factors, and also amino acids, added as Bacto vitamin-free Casamino Acids (Difco Manual, 1953, p. 230). At one stage the medium was made up synthetically, the 15 amino acids in Bacto amino acid assay medium being added instead of the vitamin-free Casamino Acids (a total hydrolysate of casein): but since this did not change the growth of the organisms this practice was discontinued. Because strain Pf 1 showed very moderate growth in this medium, it was modified with respect to its organic acid and carbohydrate content, and the composition of the modified medium was as follows. The concentrations are in g/l.: Bacto vitamin-free Casamino Acids, 0.12; dextrose, 0.20; malic acid, 0.8; citric acid, 0.10; uracil, 0.01; guanine, 0.01; adenine, 0.01; *dl*-tryptophane, 0.1; *l*-cystine, 0.1; biotin, 0.0000004; *p*-aminobenzoic acid, 0.0001; niacin,

0.001; riboflavin, 0.001; pyridoxin HCl, 0.002; thiamin HCl, 0.001; folic acid, 0.000012; Ca pantothenate, 0.0004; K_2HPO_4 , 0.5; KH_2PO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 4H_2O$, 0.01, with KOH to pH 4.2–4.7. To this Bacto yeast extract, 0.2, was added as desired in the experimental work.

Recently Radler (1958) recommended a particular combination of 23 amino acids for the growth of malolactic bacteria. On trial, we observed an insignificant improvement in growth compared with that in our modified medium. Radler himself noted that growth in his synthetic medium was only weak, though sufficient to cause the breakdown of considerable quantities (5 g/l.) of malic acid, observations which we confirmed.

The optimum pH for strain Pf 1 is 4.5–4.7, and in most of the experiments the pH was in this range. Further details are given as they arise.

RESULTS

Growth responses to additives, including yeast extract. The modified medium offered a favourable basis for attempts to improve the growth of our organism. There was no discernible influence when further pure substances were added, e.g. vitamin B_{12} or diphosphopyridine nucleotide or adenosine triphosphate which, according to Korkes & Ochoa (1948) and Jerchel, Flesch & Bauer (1956), accelerate malic acid breakdown by cell-free 'malic enzymes'. Other additives such as Mn^{++} and Mg^{++} ions were also ineffective. On the other hand, addition of Bacto yeast extract greatly stimulated the multiplication of the bacteria (Fig. 1); in fact there was a similar

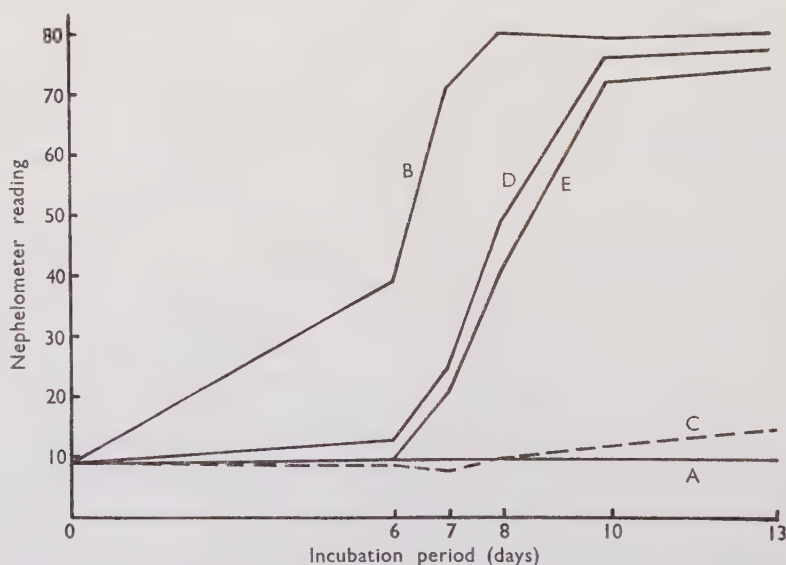


Fig. 1. Promotion of the growth of the malolactic organism Pf 1 by fractions from yeast extract.
A, modified B318 medium (control); B, Bacto yeast extract added; C, ethanol precipitate added; D, ethanol filtrate fraction added; E, both precipitate and filtrate added.

effect on all our acid-decomposing strains, though its magnitude varied from one to another. Rich & Stern (1956) published a detailed analysis of the amino acid and vitamin content of Bacto yeast extract, from which it appears that no previously known growth substance was missing from our synthetic medium. The strong growth-promoting effect of yeast extract must therefore have been caused by other components whose identity we sought to decide.

Yeast extract ash. It had first to be decided whether the stimulating component was organic or inorganic. The yeast extract was thus incinerated; but when tested the ash had practically no effect on growth, whereas the total yeast extract was very stimulating. These extract experiments, which were carried out in grape juice, therefore showed that the growth promoting activity must be caused by organic compounds.

Yeast extract hydrolysate. In further experiments, an acid hydrolysate of 'Roche' casein was used. It has already been stated that the Difco total hydrolysate 'Casamino Acids' has no growth promoting effect. This was substantiated with our own total hydrolysate. In one experiment casein (Roche) was hydrolyzed in 5 N HCl at 80–82° for periods ranging from 1 hr to 36 hr, the hydrolysates evaporated under vacuum, the residues taken up in distilled water and neutralized with NaOH. When tested in our medium the 1 and 12 hr hydrolysates promoted growth, but there was a decrease in effectiveness with increasing degree of hydrolysis, and the 36 hr hydrolysate was inactive (Fig. 2). The best stimulation was achieved with 1 hr hydrolysis: it was of the same order as that produced by yeast extract. All later experiments

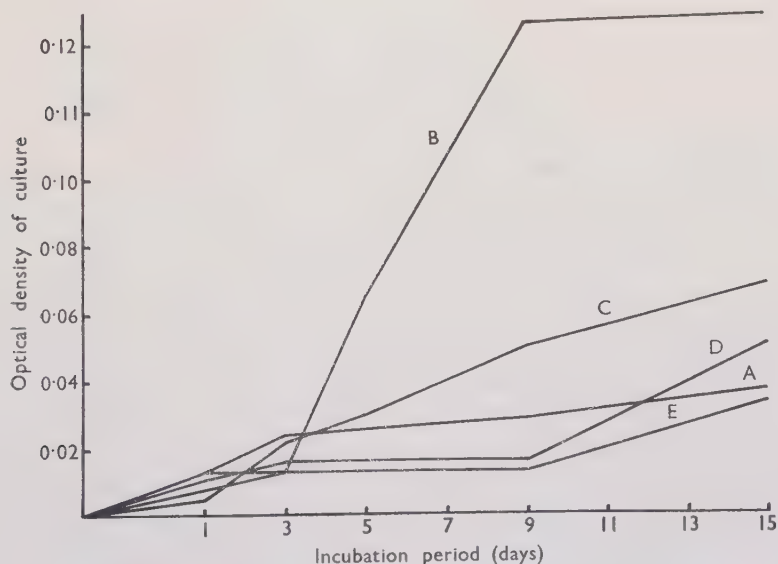


Fig. 2. Promotion of the growth of the malolactic organism Pf 1 at 22° in modified B318 medium by partial hydrolysates of vitamin-free casein (Roche). A, whole casein added; B, casein hydrolyzed 1 hr added; C, 12 hr hydrolysate added; D, 36 hr hydrolysate added; E, control, with Casamino Acids.

showed a progressive loss of activity with increase in time of hydrolysis, with both yeast extract and casein peptone.

It was therefore concluded that the growth promoting components might well be higher molecular weight nitrogen compounds. This assumption has often been made in the literature, but just as often discarded (see e.g. Radler, 1958). Further experiments were therefore necessary to resolve the issue.

If higher nitrogen compounds, e.g. peptides, were utilized during the growth of the bacteria this should be demonstrable by amino acid determinations after hydrolysis. Tests of our culture media were without success, however, because the decrease in the total nitrogen content was very slight. Analyses before and after growth, in our synthetic and semisynthetic media with nitrogen contents of 672 and 1155 mg/l. respectively, gave differences of approximately 100, i.e. 8.5%; while in the nitrogen rich medium, Difco 320, used in the early work, the difference was only 2.5%. Similarly, there were only insignificant differences in the formaldehyde number. In three experiments this fell by no more than 2–2.5 units, from the level of 20–22.5 units in a sterile control. From these experiments, it appears that the bacteria used only small quantities of the simpler nitrogen compounds such as amino acids.

Paper chromatography experiments. These were made in the hope of detecting the utilization of particular substances. But direct paper chromatography offers little aid if the nitrogen requirements are not restricted to a few compounds and, in our experiments, it was not possible to detect any disappearance of individual amino acids before or after hydrolysis. We also tested several wines in which we had allowed the malic acid to decompose completely. They had distinctly lower nitrogen contents in the range 100–800 mg/l., but usually 150–200 mg/l., and were therefore advantageous for chromatographic studies; but under these conditions too there was no evident change in concentration of any individual amino acid. These negative results indicate that our organism had no special requirements for specific amino acids and could make do with very small quantities.

Precipitation of yeast extract with alcohol. Precipitation with alcohol divided the yeast extract into two fractions whose growth stimulating effects could then be studied separately. With four parts of 96% ethanol to one of water a heavy precipitate was formed, and determinations of total nitrogen gave the following results (mg N/g): total yeast extract 121.5, alcohol precipitate 26.6, alcohol soluble fraction 98.2; difference +3.3, or 2.9%. Numerous repetitions confirmed that the major portion of the activity remained in the filtrate (Fig. 1). It appeared however that the precipitate also had some activity, though slight, which was increased when larger quantities of alcohol were used.

From these results, the growth promoting substances of the yeast extract cannot be of high molecular weight, and since there was precipitation of a small portion by means of high alcohol concentrations it may be assumed that they are of intermediate molecular size. This assumption was substantiated by the results of a dialysis experiment: during 2 days dialysis against flowing water in a cellophane tube the preparation lost two-thirds of its activity.

Ion exchange experiments. These were done in an attempt to characterize the growth promoting components of the yeast extract more closely. The weakly acidic

cation exchange resin IRC 50 was first chosen. The yeast extract was freed of its high molecular weight proteins by alcohol precipitation, as just described, and the filtrate, containing the major part of the activity, was treated with resin to remove basic amino acids. Both effluent and eluate were then tested for activity in growth experiments (Fig. 3). The chief activity remained in the effluent, the eluate (basic amino acids) having only slight activity, as might be expected. However, it was noted that the two fractions when recombined had practically the same activity as the original filtrate (compare Figs. 1 and 3).

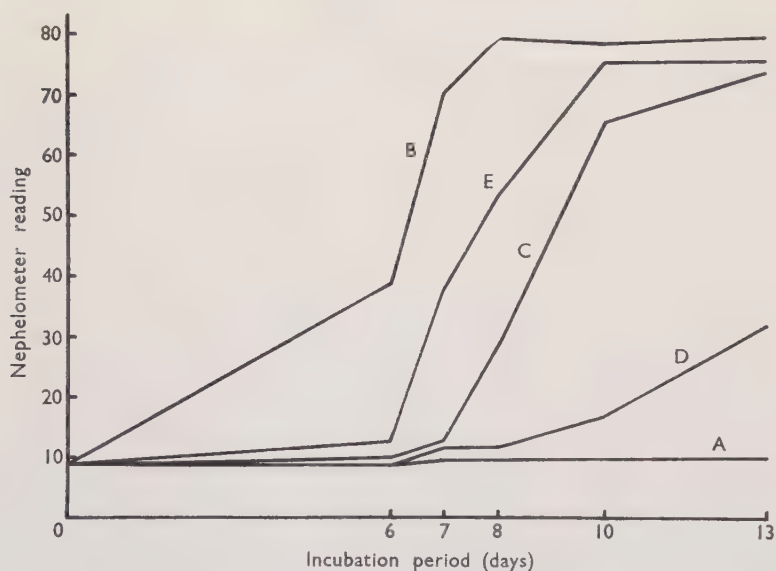


Fig. 3. Promotion of the growth of the malolactic organism Pf 1 at 22° by fractions derived from the ethanol-soluble portion of Bacto yeast extract by treatment with IRC 50 ion exchange resin. A, modified B318 medium (control); B, yeast extract added; C, effluent added; D, eluate added; E, both effluent and eluate added.

With the strongly acid cation exchanger Dowex 50 (50–100 mesh) the situation was more complex, for this resin retained high molecular weight nitrogen compounds, as proved by its behaviour towards total and partial protein hydrolysates (Table 1). A check of the nitrogen balances between effluent and eluate showed that whereas the constituents of a total hydrolysate (Casamino Acids) and of the alcohol soluble fraction of a partial hydrolysate (Proteose peptone) suffered virtually no loss (1–2% in numerous experiments) in passing through the column, with whole Proteose peptone about 25% of the nitrogen could not be eluted with ammonia; but this evidently belonged to the alcohol insoluble high molecular weight fraction, which as shown earlier does not contain the major growth promoting activity. The fact that the resin retained small quantities of simpler peptides which could be eluted with ammonia was shown by paper chromatography after hydrolysis of the eluate: there was only a small increase of a few amino acid spots.

Table 1. Nitrogen balances for the fractions derived from different hydrolysates by treatment with the strong cation exchange resin Dowex 50

Fraction of hydrolysate examined	Total N (mg/10 ml) in fractions from hydrolysates		
	Complete (Casamino Acids)	Partial (Proteose peptone)	
		Alc. sol. frac.	Whole
Original solution	16.70 (100%)	98.2 (100%)	24.08 (100%)
Effluent from resin	0.14 (0.8%)	12.6 (12.8%)	4.05 (16.9%)
NH ₃ eluate from resin	16.35 (98.0%)	87.5 (89.0%)	14.07 (58.5%)
Losses	0.21 (1.2%)	-1.9 (-1.9%)	5.96 (24.6%)

The growth experiments with yeast extract treated on Dowex 50 columns are summarized in Fig. 4. The ammonia eluate had considerable stimulating activity; that of the effluent was slight. This too suggests that the growth promoting substances of yeast extract are of relatively low molecular weight. With this resin however, unlike IRC 50, effluent and eluate combined did not have the activity of the original solution: if not due to a loss, this might be the result of hydrolysis on the strongly acid resin.

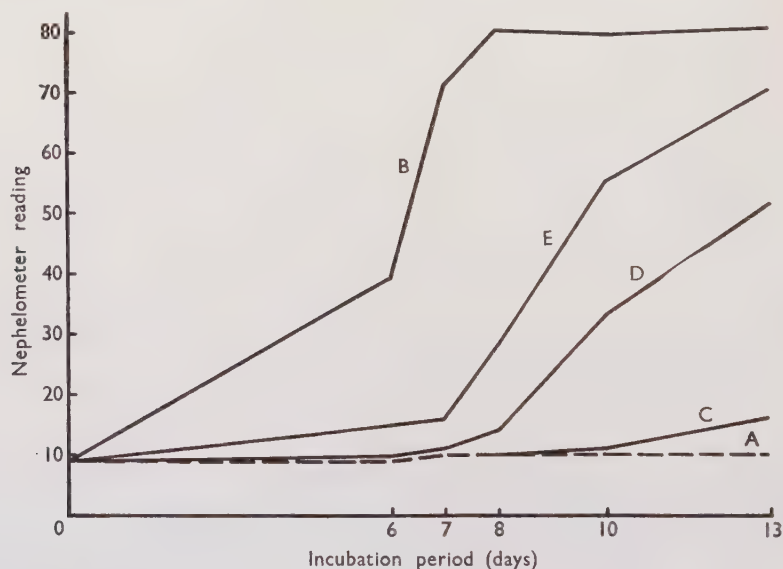


Fig. 4. Promotion of the growth of the malolactic organism Pf 1 at 22° by fractions derived from the ethanol soluble portion of Bacto yeast extract by treatment with Dowex 50 ion exchange resin. A, modified B318 medium (control); B, yeast extract added; C, effluent added; D, eluate added; E, both effluent and eluate added.

Concentration of the growth promoting substances. In the latest experiments an attempt was made to concentrate the active substances by paper chromatography. The yeast extract was applied heavily along the starting line and the chromatogram was developed for 16 hr with butanol-acetic acid-water (8:2:2). The paper was cut

into strips, and the eluates from these strips were used in growth experiments. The greatest activity was in the two first strips with Rf 0.17-0.38. Later strips showed practically no activity. We hope to go on from this point to characterize the active substance more completely.

DISCUSSION

The experiments described above show clearly that there are unknown substances in yeast extract and Proteose peptone which stimulate bacteria responsible for the malolactic fermentation. It appears that the active components are simple nitrogen compounds, perhaps peptides. Further, when a strepogenine-active substance (Hoffman-La Roche, Basel) was incorporated, decidedly better growth was obtained, though the activity was not as great as that of the Dowex 50 eluate, perhaps because of differences in effective concentration. It thus seems possible that the growth promoting agents in yeast extract are strepogenine-active substances.

The authors are indebted to Dr. M. Ingram for his assistance in the preparation of this paper.

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(Symposium on The Lactobacilli: Paper VIII)

STUDIES ON LACTOBACILLI CAUSING ROPINESS IN BEER

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SUMMARY: Three strains of lactobacilli isolated from ropy beers closely resembled *L. brevis* but differed from it in possessing a capsule and in their ability to induce ropiness in liquid cultures. Using a new technique for estimating ropiness, it was found that attainment of the ropy state in semidefined media depended on the presence of an optimal concentration of acetate and of an excess, relative to growth requirements, of a suitable source of carbon. Whereas extracellular nucleic acid was found in some only of the ropy cultures, polysaccharide (of mainly glucose units) was present in all. However, the degree of ropiness did not depend solely on the amount of polysaccharide produced. It was reversibly affected by alteration of the final pH value of the culture and possibly involved the direct participation of other substances.

ROPINESS in beer, in which the normally effervescent liquid becomes characteristically viscous and pours as an 'oily' stream, is a comparatively rare form of spoilage resulting from microbial action. While more usually associated with the growth of species of *Acetobacter* or *Pediococcus*, it may also result from infection with certain strains of *Lactobacillus*. Shimwell (1949) described a rope-forming organism isolated from ropy bottled beer as a capsulated variant of *Lactobacillus pastorianus*. Unfortunately this culture lost its ability to produce rope before his observations were completed and since then there has been no systematic study of this type of organism. The present communication describes the properties of three strains of *Lactobacillus* capable of producing ropiness in beer. Several apparently similar, but physically distinguishable, conditions may be included under the general heading of ropiness and it is desirable to describe as accurately as possible the characteristics of the particular ropy fluids referred to, for terms such as 'thick' or 'treacly' may be inadequate or misleading. Thus the beers examined by the author did not display the 'lumpy' character of beers made ropy by *Acetobacter* species (Shimwell, 1947) while their Newtonian viscosity was no greater than that of their non-ropy counterparts, a feature which clearly distinguished them from the truly viscous 'oily' ciders discussed by Millis, Husain, Hall & Walker (1958). The ropy beers and culture fluids described in this communication were characterized by their 'oily' pouring properties, by their relatively low Newtonian viscosity, and by the fact that they could be pulled out in long and rather elastic threads simply by inserting and withdrawing a suitable rod.

MATERIALS AND METHODS

Organisms and cultural methods

Organisms. Three strains were used. Two of these, A and B, came from ropy beers, the latter being kindly provided by Dr. J. O. Harris, and the third, strain C, was a mutant which arose in a laboratory culture of a non-rope-forming beer spoilage *Lactobacillus* which was being maintained by serial subculture in beer.

Yeast autolysate. This was prepared by the method of Millis *et al.* (1958).

Amino acid solution. A concentrated mixture of amino acids, which was used as a component of several media, was prepared by autoclaving a mixed solution in distilled water of Difco 'Casamino Acids' (20.0%, w/v), DL-cystine (0.4%, w/v) and DL-tryptophane (0.4%, w/v) at 5 lb/in² for 15 min.

Semidefined media. The complete medium used for certain nutritional tests was based on that of Chamberlain & Rainbow (1954) and 100 ml contained: maltose, 2.4 g; amino acid mixture, 5.0 ml; NH₄Cl, 0.25 g; CH₃COONa.3H₂O, 3.0 g; NaCl, 0.4 g; KCl, 0.075 g; Na₂HPO₄, 0.05 g; NaH₂PO₄, 0.05 g; MgCl₂.6H₂O, 0.008 g; MnCl₂.4H₂O, 0.5 mg; FeCl₃.6H₂O, 0.04 mg; adenine, guanine, thymine, uracil and xanthine, 1.0 mg each; thiamine, pyridoxine, nicotinic acid, calcium pantothenate and riboflavin, 0.1 mg each; biotin, 0.04 mg; *p*-aminobenzoic acid, 0.02 mg; folic acid, 0.01 mg. Growth on this medium was relatively slight, and it was used only for testing the vitamin requirements of the three organisms. For all other purposes, much better growth was obtained by omitting the vitamins and incorporating Difco yeast extract (0.2%, w/v).

Inoculation and incubation. For the preparation of inocula, stock cultures maintained at 4° on solid medium L (Williamson, 1959) were streaked on the same medium and after incubation for 3 days the actively growing cultures were harvested and the cells washed and suspended in sterile saline. Cultures on solid medium L and in the liquid semidefined medium were incubated in desiccators filled with carbon dioxide. All other cultures were incubated in tightly screw-capped bottles filled to the neck with the appropriate medium. Unless otherwise indicated, the incubation temperature was 25°.

Biochemical tests used in classification

Heat resistance. The ability of the organisms to survive at 60° for 90 min and (where necessary) 65° for 30 min was tested by the method of Briggs (1953) using a liquid version of medium L (Williamson, 1959).

Salt tolerance. Ability to grow in the presence of 2.0%, 4.0% and 6.0% (w/v) of NaCl (Briggs, 1953) was tested by incubating the organisms for 14 days in a medium consisting of yeast autolysate (5.0%, v/v), maltose (1.0%, w/v) and the amino acid solution (5.0%, v/v), plus NaCl in the desired concentrations.

Growth temperatures. The organisms were incubated in a liquid version of medium L (Williamson, 1959) for 14 days at 15° or 45° in screw-capped bottles in an accurately controlled water bath.

Production of ammonia from arginine. Cultures were incubated for 7 days in a medium consisting of yeast autolysate (5.0%, v/v), maltose (1.0%, w/v) and arginine

(0.3%, w/v). After centrifuging, 1.0 ml samples of the supernatant were mixed with an equal volume of Nessler's reagent and the resulting colour compared with that given by a similar sample from a control culture in the medium without the arginine.

Carbohydrate fermentations. The basal medium contained yeast autolysate (5.0%, v/v) and the amino acid mixture (5.0%, v/v), the appropriate carbohydrate being added in 1.0% (w/v) concentration. After adjusting to pH 6.5 and adding bromocresol purple, most of the media were dispensed into bijou bottles with Durham tubes and autoclaved at 5 lb/in² for 15 min; but media containing ribose, xylose, arabinose, galactose or fructose were sterilized by Seitz filtration. Cultures were incubated at 30° for 17 days and examined daily for production of acid and gas.

Hydrolysis of aesculin. Cultures in the medium of Davis (1955) were incubated at 30° and examined daily for 5 days for blackening and loss of fluorescence.

Catalase. Catalase production was tested for by flooding 5-day cultures on solid medium L with 100-volume hydrogen peroxide and examining for the evolution of gas.

Acid production. Cultures incubated for 7 days in the semidefined medium containing maltose (2.4%, w/v) were centrifuged and portions of the supernatant titrated against 0.1 N NaOH using phenolphthalein as indicator. The lactic acid content of the culture supernatant was determined by the microdiffusion method of Ryan (1958).

Analytical methods

Isolation of extracellular polysaccharide. The amount of diffusible extracellular polysaccharide produced by the organisms growing in beer or in defined media was generally small and isolation of the material from cultures in beer was complicated by the presence of non-dialysable dextrans. These difficulties were overcome by growing the organisms inside a sterile sac of dialysis tubing suspended in a large volume of medium, the sac being initially filled with a sterile solution of yeast extract (0.3%, w/v). After about 10 days incubation the sac was removed, the concentrated culture centrifuged and the supernatant liquid dialyzed against running tap water for 3–4 days, a few drops of toluene being added to prevent bacterial growth. Two volumes of ethanol were added with stirring at 4° and the resulting precipitate was washed several times in 70% (v/v) ethanol. It was then redissolved in distilled water and reprecipitated with two volumes of ethanol. Finally, the precipitate was dissolved in a small volume of distilled water and freeze dried.

Paper chromatography. Samples of polysaccharide were completely hydrolyzed by digestion for 3 hr at 100° in 2 N sulphuric acid. After neutralization with barium carbonate the supernatant liquid was concentrated on a boiling water bath. Descending chromatograms were run on Whatman no. 1 paper overnight, using a mixture of ethyl acetate, pyridine and water (10:4:3). Separation of fructose and mannose was accomplished by means of the ethyl acetate-acetic acid mixture of Jermyn & Isherwood (1949), the development time in this case being 3 days. Specific reagents used for the location and identification of the sugar spots included naphthoresorcinol, diphenylamine (Harris & MacWilliam, 1954) and aniline oxalate (Horrocks & Manning, 1949).

Estimation of extracellular polysaccharide and nucleic acid. Cultures in the semi-defined medium were centrifuged (17,000 g) for 30 min and the supernatant liquid removed. The deposited cells were then washed once by centrifugation in distilled water, the washing water being combined with the first supernatant. The pooled supernatants were dialyzed against running tap water for 3 days and the total non-dialysable carbohydrate content of suitably diluted samples was determined by the anthrone method of Hall (1956). An approximate estimate of the presumed nucleic acid content of the dialyzed supernatant was obtained by ultraviolet absorption spectroscopy. The complete spectrum was examined in the range from 220 m μ to 280 m μ and the absorption at 260 m μ of those samples showing the typical spectrum of nucleic acids was compared with that of a commercial preparation of 'yeast nucleic acid'.

Estimation of growth. The total growths of cultures were estimated turbidimetrically using a Spekker photoelectric absorptiometer and were expressed in arbitrary units. Concentrated cultures were diluted so that readings lying on the linear part of the curve relating turbidity to concentration were obtained.

Estimation of viscosity and of ropiness. An arbitrary estimate of the Newtonian viscosities of culture fluids was obtained by measuring the time of outflow through an Ostwald capillary viscometer maintained at 20°; but as shown below the degree of ropiness of the culture fluids examined was not associated with an increased Newtonian viscosity and an arbitrary but direct method of estimating ropiness, based on the length of the continuous thread that could be pulled out of the fluid, was devised. About 12 ml of the test fluid was placed in a shallow dish approximately 5 cm in diameter and 0.5 cm deep. The end of a clean test tube 19 mm in diameter was then inserted to the bottom of the dish, pulled out rapidly in a vertical direction, and the length of continuous thread pulled out measured against a scale graduated in inches. The operation was repeated several times and the mean length of the thread determined. In order to reduce subjective errors as much as possible, samples were always examined in a random order, and the results recorded here are the mean values obtained by two operators. The thread lengths ranged from less than 1 in. with an uninoculated medium to as much as 15 in. with a very ropy culture, and in this range differences in thread length of the order of 5%–20% could be distinguished. The method had the advantage of measuring directly the characteristic by which the ropy state of the fluids was recognized, and it permitted the detection of slight degrees of ropiness which might otherwise have been overlooked.

RESULTS

General characteristics of the strains

Morphology. All three strains were Gram-positive, non-motile, non-sporing catalase-negative rods, which on complex solid or liquid media were generally of medium size, about 4.0 $\mu \times$ 1.0 μ . In semidefined media filaments about 40 μ long were often formed. All three organisms were capsulated, and examination of wet mounts in india ink of cells from certain cultures revealed the presence of masses of loose drifting slime (see Plate 1).

Colony characters. Colonies on medium L were greyish, opaque, circular, entire and raised with a smooth glossy surface. By means of a loop they could be pulled out in a characteristic elastic thread, and if the medium was very dry the entire colony sometimes 'jumped' on to the loop; more usually it was extremely difficult to detach from the surface of the medium.

Growth in liquid media. This was at first diffuse, but later the organisms sedimented and formed a mass which on gentle rotation of the bottle spiralled upwards as a thick mucoid 'string'. With few exceptions, liquid cultures were ropy.

Table 1. *Biochemical characteristics of three strains of lactobacilli causing ropiness in beer*

Character	Behaviour* of strain		
	A	B	C
Ratio lactic acid:total acid (%)	72	55	66
Ammonia from arginine	+	+	+
Heat survival { 60°, 90 min	+	+	—
{ 65°, 30 min	—	+	n.t.
Growth at { 15°	+	+	+
{ 45°	—	—	—
Maximum NaCl concentration (% w/v) tolerated	2.0	2.0	2.0
Catalase production	—	—	—
Hydrolysis of aesculin	+	+	+
Fermentation† of: fructose	A	A	A
galactose	A	NC	A
glucose	A	A	A
maltose	A	A	A
mannitol	W	W	W
melibiose	A	A	A
raffinose	AR	AR	AR
ribose	A	A	A
xylose	A	A	A

* A, acid without visible gas; NC, no change; AR, acid produced but pH returned to neutrality in a few days; W, acid production doubtful; n.t., not tested.

† None of the strains produced acid from arabinose, cellobiose, dulcitol, ethanol, glycerol, inositol, lactose, melezitose, salicin, sucrose or trehalose.

Biochemical characteristics. The biochemical characters of the organisms recorded in Table 1 show that all three strains were heterofermentative, as lactic acid never accounted for more than 72% of the total acid produced from maltose. It may also be mentioned that copious gas production was sometimes observed in cultures in liquid synthetic media containing maltose, though this behaviour was variable.

Nutritional requirements. The requirements of the three organisms for certain organic growth factors are shown in Table 2. In common with other lactobacilli from brewing materials (Moore & Rainbow, 1955; Russell, Bhandari & Walker, 1954) all three strains displayed an absolute requirement for pantothenate and nicotinic acid. They also required amino acids for growth, but their precise requirements in this respect were not established.

Table 2. *The growth factor requirements of three strains of lactobacilli causing ropiness in beer*

Growth factor	Requirement* of strain		
	A	B	C
Thiamine	S	O	O
Pyridoxine	S	O	S
Nicotinic acid	E	E	E
Pantothenate	E	E	E
Riboflavine	O	O	S
<i>p</i> -Aminobenzoic acid	O	O	O
Folic acid	E	O	E
Biotin	S	O	E
Adenine	S		E
Guanine	S	O	E
Uracil	E	S	E

* O, not essential, omission led to little or no reduction of growth;
S, stimulatory, omission reduced growth to 30–60% of control;
E, essential, omission reduced growth to 30% of control.

The nutritional characteristics of strains B and C were not investigated further, but strain A was found to require acetate for the assimilation of sugars, and only certain of these supported growth. The data in Fig. 1 show that the heaviest crops were obtained on maltose and fructose, which were also the only two sugars giving rise to significant ropiness. In its inefficient utilization of glucose for growth, this strain was similar to the lactobacilli from brewing sources described by Moore & Rainbow (1955).

Chemical composition of the extracellular non-capsular slime. Complete hydrolysis of the polysaccharides isolated from the three strains grown as described above in

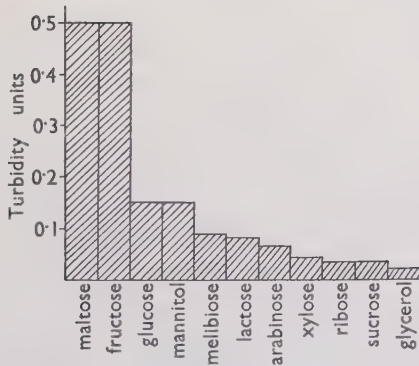


Fig. 1. The amounts of growth of strain A supported in the semidefined medium by different carbon sources, each at a concentration of 0.3% (w/v).

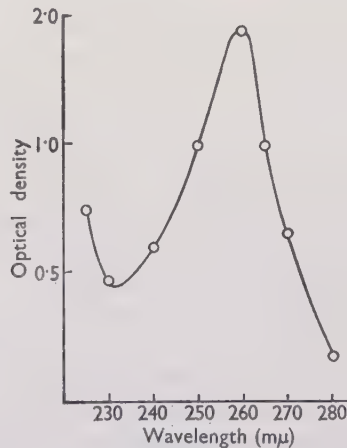


Fig. 2. The ultraviolet absorption spectrum of dialyzed supernatant fluid of a culture of strain A in semidefined medium containing 2.4% (w/v) of maltose.

beer enriched with yeast extract (0.3%, w/v) yielded mainly glucose units. Traces of fructose and mannose were also present but these may have resulted from contamination with whole cells or constituents of the medium.

The dialyzed supernatant fluids of certain cultures of strain A (described below) were found to have the ultraviolet absorption spectrum characteristic of nucleic acids (Fig. 2). But since the diphenylamine method for estimating deoxyribose (Burton, 1956) revealed the absence of deoxyribonucleic acid the absorbing material was thought to be ribonucleic acid. Unfortunately application of the orcinol technique for pentose (Mejbaum, 1939) did not confirm this, probably because interfering substances, possibly the contaminating polysaccharide, prevented the development of the colour.

Examination of the dialyzed supernatant fluids of several cultures of strain A using the method of Lowry *et al.* (1951) revealed the absence of protein, and this was supported by the finding that the amounts of nitrogen present were no greater than would be consistent with the observed concentrations of nucleic acid. Accordingly, it was concluded that the extracellular slime of this strain usually consisted of a dextran-like polysaccharide, nucleic acid being present only in certain cultures. It should however be emphasized that all the fluids were dialyzed prior to analysis and, as will be discussed later, it remains possible that this procedure might have resulted in the loss of other significant components of the slime.

Observations on rope formation

The independence of ropiness and Newtonian viscosity. Early in the course of this work it became clear that the ropy state of the cultures under investigation could not be assessed by measuring their Newtonian viscosity. The Ostwald capillary viscometer and a Höppler 'rolling ball' instrument have both been used to examine a large number of cultures exhibiting a wide range of ropiness, and no culture has yet been found to possess a true viscosity significantly greater than that of the uninoculated medium. This interesting situation is not unique to the ropy beers and culture fluids described here. It has also been observed in ropy wines, and some of the methods devised to assess the degree of ropiness of the latter have been discussed by Lüthi (1953) and by Hochstrasser (1955). Most of these methods are extremely tedious, and even had they been directly applicable to the present work the simple method of measuring ropiness described above seemed preferable for observations involving a large number of measurements.

The influence of maltose concentration on ropiness. It was anticipated that incorporation in the medium of more maltose than was required for the maximum growth supported by the other constituents would lead to an increased production of extracellular polysaccharide and hence to an enhanced degree of ropiness. In order to test this supposition strain A was grown for 7 days in semidefined media containing 0.1% (w/v) of sodium acetate (a concentration shown below to be about optimal for rope formation) and varying concentrations of maltose. The results (Fig. 3) showed that whereas increasing the amount of maltose to about 0.8% (w/v), i.e. to a level just in excess of the minimum required for full growth, led to a markedly

greater degree of ropiness, additional increases in maltose concentration had little further effect. Chromatographic examination revealed the presence of residual free maltose in all the cultures containing initially more than 0.7% (w/v) of the sugar, so that the upper limit to ropiness could not have been set by exhaustion of the carbon and energy source preventing the formation of further polysaccharide. In any case, the data in Fig. 3 show that there was no simple correlation between the concentration of polysaccharide and the degree of ropiness. The same was true of the nucleic acid component of the slime, for this was only formed in the culture containing the largest amount of maltose and was clearly not essential to the attainment of the ropy state. It can only be concluded that some factor (or factors) undetermined was influencing the degree of ropiness independently of the amount of polysaccharide present. Possible explanations are discussed in more detail below.

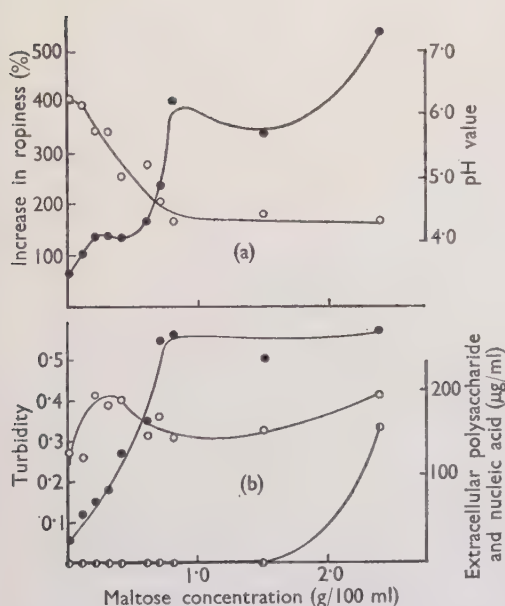


Fig. 3. The influence of maltose concentration on: (a) final pH value (open circles) and ropiness (closed circles); (b) growth (closed circles), formation of extracellular polysaccharide (open circles) and nucleic acid (half-full circles).

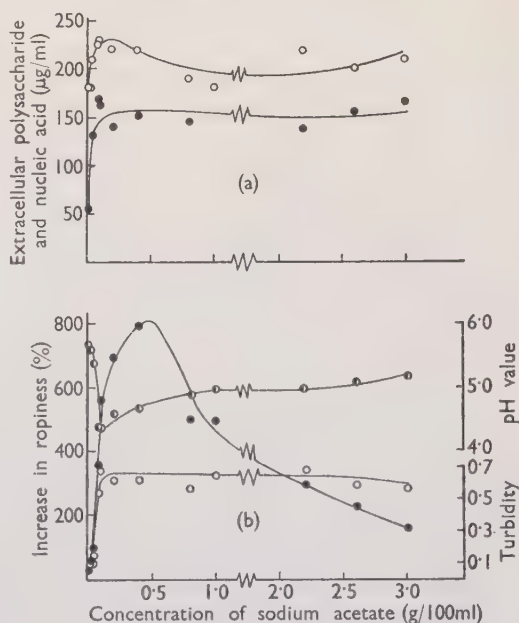


Fig. 4. The influence of sodium acetate concentration on: (a) production of extracellular polysaccharide (open circles) and nucleic acid (closed circles); (b) growth (open circles), ropiness (closed circles) and final pH value (half-full circles).

The effect of acetate concentration on ropiness. The results obtained with cultures of strain A grown for 7 days in semidefined media containing maltose (2.4%, w/v) and different amounts of acetate are shown in Fig. 4. Several features are of interest. Acetate was evidently necessary for both growth and rope production; but whereas the total growth was unaffected by a relative excess of acetate, such an excess markedly inhibited the development of ropiness. Moreover, as in the observations

on maltose concentration (Fig. 3), the amounts of extracellular diffusible polysaccharide and nucleic acid were not directly correlated with the degree of ropiness, which again suggested the existence of some undetermined factor.

The influence of the final pH value of the medium on ropiness. It was noted that there was a slight tendency for the degree of ropiness of the cultures to vary inversely with their final pH values. Accordingly, a ropy culture was centrifuged, samples of the supernatant were adjusted to different pH values, and the degree of ropiness of each sample was measured. The results (Fig. 5) show that the degree of ropiness was rather more than doubled on reducing the pH from 7.0 to 4.0. This change was completely reversible, which indicated that the effect of pH was physical rather than chemical in nature. It should be made clear that this effect related to the final pH value of the medium only; the influence on ropiness of the initial pH value of the medium, which is presumably more complex, was not examined.

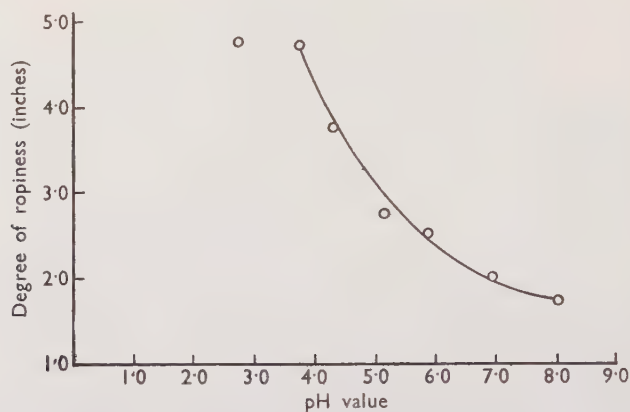


Fig. 5. The influence of the final pH value of the medium on the degree of ropiness.

It is apparent that the final pH value of the medium would influence the degree of ropiness attained. Nevertheless the magnitude of the pH effect would appear to be too small entirely to account for the observations recorded in Figs. 3 and 4, and some alternative explanation of the apparent discrepancy between polysaccharide concentration and ropiness must be sought. Some possibilities are discussed below.

DISCUSSION

The heterofermentative nature of the three strains, together with their ability to grow at 15° and their failure to grow at 45°, clearly places them in group VII of Briggs (1953) and indicates a relationship to the heterofermentative mesophilic species *Lactobacillus brevis*, *L. buchneri* and *L. pastorianus*. Confirmation of this grouping was obtained by Dr. M. E. Sharpe of the National Institute for Research in Dairying, who kindly submitted the cultures to serological examination and found that all three reacted specifically with group E serum (Sharpe, 1955; Sharpe & Wheater, 1957).

Of the named species included in group VII, the present organisms most closely resemble *L. brevis* as described by Rogosa *et al.* (1953) and are distinguished from *L. buchneri* by their ability to hydrolyze aesculin and their failure to ferment melezitose. So far as comparison is possible, they do not resemble *L. pastorianus* any more closely, though as pointed out by Shimwell (1957) this species is rather ill-defined and the available descriptions are inadequate for precise identification. In these circumstances the present organisms are best regarded as capsulated variants of *L. brevis*.

The condition of ropiness observed in cultures of strain A was evidently of a complex nature. While it may reasonably be assumed that the extracellular polysaccharide formed during growth was an essential feature of the condition, the influence of factors other than the amount of polysaccharide was indicated by the absence of a direct correlation between the latter and the degree of ropiness. One such possible factor was the presence of encapsulated bacterial cells, for although there was no close relationship between the amount of growth and the degree of ropiness the latter was invariably reduced somewhat by centrifuging the cultures. Nevertheless, the very existence of marked ropiness in cell-free culture fluids showed that the synthesis of diffusable extracellular materials was an important factor. Macromolecular substances may well be implicated in ropiness, but of the likely alternatives only polysaccharide was invariably present, while extracellular nucleic acid was absent from several ropy cultures and protein was never detected. However, it may be significant that all the chemical analyses were done on fluids which had been dialyzed for several days at room temperature, and as it has recently been observed that dialysis always results in a marked loss of ropiness which cannot be restored by adjusting the pH value of the fluid, it is possible that dialysis entails the loss of some essential component of the ropy system. If this component were a macromolecular substance it would supposedly have suffered degradation during dialysis into dialyzable units of low molecular weight, perhaps through the activity of extracellular enzymes.

Alternatively, the ropy fluids may include dialyzable compounds capable in some way of enhancing the degree of ropiness. Although a detailed discussion of possible mechanisms is beyond the scope of this communication, the reversibility of the variations in ropiness brought about by altering the pH value of the supernatant fluid suggests that the manifestation of ropiness depends on a physical aggregation of the polysaccharide molecules. If this is so, it may be that certain dialyzable compounds (e.g. salts) enhance the degree of ropiness by forming an easily dissociated complex with the polysaccharide. To account for loss of ropiness on dialysis, the further assumption is required that the hypothetical complex is in equilibrium with free molecules of the dialyzable component.

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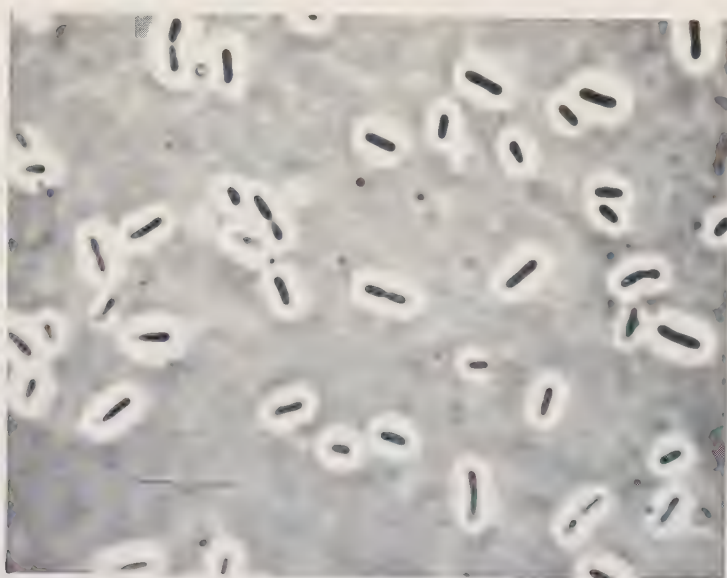
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EXPLANATION OF PLATE

Phase contrast photomicrographs of cells of strain A suspended in india ink.

- (a) 5-day culture on solid medium L showing well marked capsules.
- (b) 7-day culture in a semidefined medium containing 0.1% (w/v) of sodium acetate and 2.4% (w/v) of maltose. In addition to the capsules, ill-defined patches of loose drifting slime are visible.

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(a)



(b)

KEDDIE, R. M. (1959). *J. appl. Bact.* **22** (3), 403-416.

(Symposium on The Lactobacilli: Paper IX)

THE PROPERTIES AND CLASSIFICATION OF LACTOBACILLI ISOLATED FROM GRASS AND SILAGE

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SUMMARY: The characteristics of 61 representative strains of lactobacilli isolated from herbage and from laboratory silage are described. Most isolates of the low temperature homofermentative group were identified as *Lactobacillus plantarum*, but about 50% of the strains attached to that species were atypical mainly in lacking the ability to ferment maltose and mannitol. Other isolates included several strains of a type similar to motile lactobacilli reported to occur in silage made by the AIV method. *L. casei* was isolated on few occasions. Most of the high temperature homofermentative isolates were identified as *L. acidophilus* and the remainder resembled *L. salivarius* most closely. Heterofermentative strains represented a notable proportion of the isolates: most were attached to *L. fermenti*. Less frequently isolated were strains with characters intermediate between those of *L. buchneri* and *L. fermenti*. *L. brevis* was not encountered in laboratory silage: low temperature heterofermentative strains were isolated infrequently and were of unusual types.

A medium for detecting malate decarboxylation by lactobacilli using the agar closure technique is described. Data are presented which suggest that whereas the ability to decarboxylate malate is a usual feature of the low temperature homofermentative group, it is probably uncommon in the high temperature homofermentative group, with *L. salivarius* an exception. Most heterofermentative isolates liberated CO₂ from malate.

ALTHOUGH lactobacilli have long been considered important in the acidification of silage made from many different crops, information on the types multiplying during the early period of the fermentation is meagre. From the available data it would appear that *L. plantarum* becomes the dominant species in silage made from grass (Allen & Harrison, 1936; Allen, Harrison, Watson & Ferguson 1937), beet and cabbages (Orla-Jensen, 1947), and from lucerne (Orla-Jensen, 1947; Orla-Jensen, Orla-Jensen & Kjaer, 1947). *L. casei* in addition to *L. plantarum* has been reported to occur in grass silage (Pette, 1947), and in beet-pulp silage (Olsen, 1951). Heterofermentative species have been isolated in most cases only from material which has been ensiled for some time; *L. brevis* (Pette, 1947; Olsen, 1951) and *Betabacterium caucasicum* (Orla-Jensen *et al.* 1947) have been reported to occur in such material. It is interesting to note, however, that *L. pentoaceticus*, usually considered a synonym of *L. brevis*, was stated to be the most important species concerned in the fermentation of corn (maize) silage (Fred, Peterson & Anderson, 1921a), although types similar to *L. plantarum* were also present.

In the past, studies on the incidence of lactobacilli on herbage and in recently ensiled materials have been hampered by lack of media selective for organisms of this group. The development of suitable media (Keddle, 1951; Rogosa, Mitchell & Wiseman, 1951) has already led to the finding that lactobacilli are scarce on a wide variety of forage crops (Stirling, 1953; Kroulik, Burkey & Wiseman, 1955; Nilsson &

Nilsson, 1956). The object of the present work was to study the properties and classification of lactobacilli occurring on grass and in particular of the types that multiplied when the material was ensiled.

METHODS

Strains. Several of the strains studied were isolated from freshly harvested grass using acetate agar (Keddie, 1951), but most were representative of many hundreds isolated when the development of organisms of this group in silage made in large test tubes in the laboratory was being followed.

Silage. Laboratory silage was incubated at 20°, 30° and 40° (see Gibson, Stirling, Keddie & Rosenberger, 1958): it was made from grass of low (sterile water added) and high (wilted) dry matter content, and from minced and uncut grass. The methods used for harvesting the grass and for its ensilage have been described previously (Stirling, 1951; Gibson *et al.* 1958) and it will suffice to say here that the herbage was obtained from small plots of perennial rye-grass and that in all manipulations of grass and silage care was taken to avoid contamination from unsterile surfaces.

Routine media. These were based on Tween agar (Keddie, 1951), a medium which supported good growth of all lactobacilli encountered in this study. Unless otherwise stated Evans peptone, Lab-Lemco meat extract and Davis agar were used. Liquid and semisolid media were dispensed in 5 ml amounts in $5 \times \frac{1}{2}$ in. test tubes and sterilization was at 15 lb/in² for 15 min. The yeast autolysate and tomato extract used in most of the media were prepared by methods described elsewhere (Gibson *et al.* 1958). For media containing tomato extract, the method of preparation was arranged to give minimal heating to that constituent.

Purification and maintenance of cultures. Isolates were plated twice on Tween agar or as often as was considered necessary to ensure purity. Stock cultures were maintained in cooked meat medium in bijou bottles at room temperature. At intervals of approximately 3 months, stock cultures were subcultured in Tween semisolid agar before re-inoculating meat medium for storage. Tween semisolid agar (TSS) has the following composition: peptone, 1 g; meat extract, 1 g; yeast autolysate, 5 ml; tomato extract, 20 ml; glucose, 1 g; Tween 80, 0.05 ml; agar, 0.1 g; tap water to 100 ml; pH 6.0.

Preparation of inocula. To ensure that cultures were in a vigorous condition, at least two transfers were made in buffered Tween broth, the second being used for inoculating the test media. Buffered Tween broth (BTB) has the following composition: peptone, 1 g; meat extract, 1 g; yeast autolysate, 5 ml; tomato extract, 10 ml; glucose, 0.25 g; K₂HPO₄, 0.5 g; Tween 80, 0.05 ml; tap water to 100 ml; pH 6.0. The inoculum used was one loopful or one drop by capillary pipette unless stated otherwise. As a rule cultures capable of growth at 45° were incubated at 37°, and others at 30°.

Preliminary tests

Morphology was determined in Gram-stained and nigrosin smears of young 30° cultures in Tween broth (TSS medium without agar).

A test for catalase was made on young glucose yeast broth cultures adjusted to pH 7 before the addition of a few ml of 5 vol. H_2O_2 . In most cases this test was done in a modified Lind's apparatus (Cunningham, 1947, p. 134). Young cultures on glucose yeast agar slopes (Gibson *et al.* 1958) were also tested for catalase production. The agar and broth media had the same basic composition.

The ability to reduce nitrate was tested for in a medium similar to Tween broth but with the glucose concentration reduced to 0.1% (w/v) and containing 0.1% (w/v) of KNO_3 . Cultures incubated for 7 days at 30° were tested for nitrite and, in its absence, for residual nitrate.

Fermentation of lactate was tested for in three media. A basal medium, containing peptone, 0.5 g; meat extract, 0.5 g; yeast autolysate, 5 ml; Tween 80, 0.05 ml; agar, 0.1 g; with tap water to 100 ml, bromocresol green and at pH 5.4: the basal medium plus sodium lactate syrup (c. 70%) to give a final concentration of 1%: and the basal medium plus 1% of sodium lactate and 0.25% (w/v) of sodium acetate, hydrated. After incubation for 14 days at 30°, the growth and reaction of the cultures in the media containing lactate were compared with those in the basal medium.

The medium for detecting synthesis of extracellular polysaccharide from sucrose contained peptone, 1 g; meat extract, 1 g; yeast autolysate, 5 ml; tomato extract, 20 ml; sucrose, 5 g; gelatin (Cox), 12 g; Tween 80, 0.05 ml; water to 100 ml; pH 6.5. Stab cultures were incubated for 14 days at 23.5°. At this temperature the medium remained solid and growth of all strains could occur.

The organisms studied were Gram-positive, catalase-negative rods and coccil rods, which did not reduce nitrate. Lactate was not fermented and none formed copious slime from sucrose. No evidence was obtained that any strain could utilize lactate in the presence of air.

Further tests

Production of CO_2 from glucose. The medium and method used was similar to that of Gibson & Abd-el-Malek (1945) but yeast autolysate (5% v/v) was substituted for the Yeastrel used by those authors. A heavy inoculum, 2 to 3 drops by capillary pipette, was used.

Colony form. Two media have been used: glucose yeast agar containing 20% (v/v) of tomato extract and a tomato agar similar to that of Kulp (1927). They gave similar results. Subsurface colonies were examined by means of a stereoscopic low power microscope and three colony types were recognized, 'S' (smooth), 'R' (rough), and 'I' (intermediate or irregular). Occasionally the variant smooth colonies described by Rogosa *et al.* (1953) and by others, were observed. These ranged from c. 0.3–0.5 mm in diameter in the media used whereas those of the 'S' type were normally c. 1–2 mm in diameter. Subsurface colonies in Tween agar with or without added acetate were invariably smooth.

Temperature relationships. The medium used was Tween semisolid agar. The approximate limiting temperatures for growth were determined for most of the cultures and tests for growth at 15° and 45° were done on all isolates. For the 45° test, cultures were incubated at $45^\circ \pm 0.1^\circ$ in tubes closed with sterile rubber bungs; cotton plugs were used in other growth temperature tests. For the higher temperatures the incubation period was 5–7 days and for the lower temperatures 2–4 weeks.

Fermentation tests. In attempts to find a reliable method, various basal media with initial pH values in the range 5.4–7.0 were tried; substrates were added as concentrated Seitz filtered solutions to give a final concentration of 0.5% (w/v). At times equivocal results were obtained when indicators were used as the sole means of detecting fermentation. It appeared also that in some cases fermentation could occur in a medium of low initial pH (5.4, indicator bromocresol green), but not in a medium of higher initial pH (7.0, indicator bromocresol purple); the reverse occurred infrequently. A method described by Rogosa *et al.* (1953), using a basal medium developed from SL agar (Rogosa *et al.* 1951), was found to be free from most of the disadvantages noted above and was adopted and used in the following way: basal medium with Tryptose (Difco) in place of Trypticase was made at double strength, distributed in 10 ml quantities in 1 oz screwcapped bottles and sterilized at 15 lb/in² for 15 min. Distilled water solutions (4% w/v) of the substrates used were sterilized by Seitz filtration and 10 ml quantities of these added to equal volumes of basal medium to yield fermentation test media with a substrate concentration of 2% (w/v) and a final pH of 6.0. These were distributed in 2 ml quantities in 5 × $\frac{1}{2}$ in. test tubes and incubated at 30° for 2 days to check sterility before they were inoculated, by capillary pipette, with 1 drop of a young culture in BTB medium. The cultures were incubated at 30° for 14 days and observed daily; the time taken for the indicator (alizarin red S) to change colour distinctly was noted and after incubation the pH of each culture was determined electrometrically. In assessing the results the pH values of cultures with added substrate were compared with those of controls with distilled water in place of substrate. A final pH in the range c. 4–5 was considered to indicate a definite fermentation (designated +); a value in the range c. 5.1–5.4 indicated that a weak fermentation had occurred (designated ⊗). In negative results the pH rarely fell below 5.8.

Acid production in milk. Separated milk was distributed in 9 ml quantities in 6 × $\frac{5}{8}$ in. tubes and sterilized in steam at 100° for 30 min on each of three successive days. Duplicate tubes were inoculated and incubated at 30° for 14 days, when they were titrated with 0.1 N NaOH, using phenolphthalein. The acid formed was calculated as lactic acid, appropriate adjustment being made for acidity of the controls.

Hydrolysis of hippurate and aesculin. The methods used for both tests were similar to those described by Rogosa *et al.* (1953) using media based on that employed for the fermentation tests. For the detection of aesculin hydrolysis, a slightly modified method proved more reliable than that described by those authors. Agar and ferric citrate were omitted from the medium; blackening on the addition of ferric chloride solution to cultures (Cunningham, 1947, p. 36) indicated a positive result. In both tests the cultures were incubated for 14 days at 30°.

NH₃ production from arginine. The medium used was similar to that of Niven, Smiley & Sherman (1942) but with the addition of 0.05% (v/v) of Tween 80, 0.1% (w/v) of agar and with an initial pH value of 6. A similar medium without added arginine was inoculated as control. Ammonia production was tested for by a spot test using Nessler's reagent. Cultures incubated at 30° were tested at 3, 5, and 8 days and those incubated at 37° at 2 and 5 days. Some strains gave poor growth in the medium used but frequently such cultures gave strongly positive reactions with Nessler's reagent.

Form of lactic acid produced. The medium used was similar to Tween broth but with the glucose concentration increased to 5% (w/v). Before sterilization, chalk (10 g) was added to 300 ml amounts of medium contained in 500 ml conical flasks. A heavy inoculum was used and the cultures were incubated for 14 days at 30°. The lactic acids were separated as the zinc salts using the method described by Cunningham & Smith (1940) and the optical rotation determined by means of a polarimeter. The zinc lactates obtained were not fractionated; thus strains reported to produce L(+)-lactic acid (which gives L(-)-zinc lactate) either produced only this isomer or produced DL-lactic acid with the L(+)-isomer in excess.

RESULTS

Tests for ability to produce CO₂ from glucose and to grow at 15° and 45°, together with the colony type, permitted the subdivision of a large majority of the isolates into five major groups. This grouping was supported by the results of other tests.

Homofermentative strains

Group 1. As shown in Table 1 the organisms of this group were capable of growth at 15° and at 10° but not at 45° (except strain 3) and produced 'S' type colonies. These characteristics are those of the low temperature homofermentative group, *Streptobacterium* Orla-Jensen (Orla-Jensen, 1919, 1943).

Table 1. *The characteristics of 17 strains of lactobacilli of group 1*

Test	Behaviour* of strain no.																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Growth at 45°	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Acid in milk (%)	1.2	1.0	1.2	0.6	0.5	0.5	0.4	0.2	0.2	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Form of lactic acid	nt	L(+)	L(+)	DL	nt	nt	nt	nt	nt	nt	nt	DL	nt	nt	nt	DL	nt
Melibiose	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	⊗†	⊗†	⊗†	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sorbose	+	+	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Maltose	+	+	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Mannitol	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Sorbitol	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Raffinose	—	—	—	+	+	+	+	+	+	—	—	—	—	—	—	—	—
Rhamnose	—	—	+	—	—	—	—	—	—	—	⊗	⊗†	⊗	—	⊗	⊗	⊗
Xylose	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+
Melezitose	+	+	+	—	—	—	+	+	+	+	—	—	—	—	—	—	—
Inulin	+	—	—	+	+	+	—	—	—	—	—	—	+	—	—	+	—
α-methyl-glucoside	+	+	—	—	+	+	+	—	—	nt	—	—	—	—	—	—	—
α-methyl-mannoside	+	—	—	—	—	—	+	+	+	nt	—	—	—	—	—	—	—
Aesculin	+	—	—	+	+	+	+	+	+	+	—	—	—	—	—	—	—
Hippurate	+	+	—	+	+	+	+	+	+	+	+	—	—	—	—	—	—
Arginine	—	—	—	—	—	—	—	—	—	—	⊗	+	+	+	+	+	+

* ⊗, weak positive reaction; †, late fermentation; nt, not tested.

No strain shows gas formation in glucose media. All strains grow at 15°. Colonies are smooth (S).

All strains ferment glucose, fructose, mannose, cellobiose, trehalose, galactose and sucrose. A large majority ferments salicin (15/17) and lactose (16/17). Strain 3 ferments dulcitol.

Strains 1–3 differed from the remainder in forming at least 1% of acid in milk, in being unable to ferment both melibiose and arabinose and in being able to ferment inositol to a limited extent, while two representative strains produced L(+)-lactic

acid. The characters of these isolates are in good agreement with those recorded for *L. casei* by Orla-Jensen (1919, 1943) and by more recent workers (Rogosa *et al.* 1953). Strain 3 differed from the others particularly in growing at 45° and in fermenting rhamnose; its characters correspond with those of the high temperature type of *L. casei* described by Orla-Jensen (1919) and are in excellent agreement with those of *L. casei* var. *rhamnosus* (group 3 strains) as described by Rogosa *et al.* (1953).

The remaining strains of this group are obviously related to *L. plantarum* (Orla-Jensen, 1943); most did not produce more than 0.5% of acid in milk, 12 out of 14 fermented melibiose and 13 out of 14 attacked arabinose. In addition only DL-lactic acid was produced by the three representative strains tested. The two strains which in repeated tests did not ferment melibiose differed only in this character and in minor respects from isolates which fermented this substrate. However, of these 14 strains only nos. 4–10 can be considered completely typical of *L. plantarum* (Orla-Jensen, 1919, 1943; Pederson, 1936). Strains 12–17 differed from typical representatives of this species in some respects and more especially in lacking the ability to ferment maltose and mannitol. Strain 11 did ferment maltose but resembled strains 12–17 in most other respects. Although a few mannitol-negative strains of *L. plantarum* have been described (Orla-Jensen, 1943; Pederson, 1936), most of the descriptions of this species indicate that it invariably ferments maltose (Orla-Jensen 1919, 1943; Rogosa *et al.* 1953; Wheater, 1955). Nevertheless the differences between strains 12–17 and the strains of *L. plantarum* described by previous workers seem to be those of varieties rather than of species. It is interesting to note that a few strains which seem similar to those just described were isolated from AIV type silage by Cunningham & Smith (1940); these authors also concluded that such strains should probably be attached to *L. plantarum*.

Group 2. The strains placed in this group (Table 2) were of the low temperature type but differed from those of group 1 in several respects. Subsurface colonies in tomato agar were small (c. 0.3 mm) and irregular to rough in form, a type more usually associated with the high temperature group. Growth in media that did not contain Tween 80 was poor. Microscopically the organisms were small, regular, slender rods and coecal rods which occurred in pairs and short chains. The rods were sometimes curved. Two strains (nos. 20 and 21) were actively motile; motility in strain 21 was by a single flagellum attached close to a pole of the cell. A narrow range of substrates was fermented and a notable feature was the inability to attack sucrose, a sugar fermented by all other homofermentative lactobacilli encountered in this study. Only one strain fermented lactose to an appreciable extent. Of three strains tested, two produced L(+)-lactic acid and the third DL-lactic acid.

It is obvious that the organisms of group 2 are distinct from *L. casei* and *L. plantarum*, the only clearly defined species of the low temperature homofermentative lactobacilli. They seemed to resemble certain of the motile lactobacilli described by Cunningham & Smith (1940) and an examination of four strains originally isolated by these authors largely confirmed this view: but two of those strains, in agreement with the original description, were found to ferment sucrose. One of the sucrose-fermenting strains further differed from the recent isolates in forming smooth colonies.

In view of these observations it would seem premature to suggest that group 2 constitutes a distinct species.

Table 2. *The characteristics of 6 strains of lactobacilli of group 2*

Test	Behaviour* of strain no.					
	18	19	20	21	22	23
Acid in milk (%)	<0.1	0.1	<0.1	<0.1	<0.1	<0.1
Form of lactic acid	L(+)	L(+)	DL	nt	nt	nt
Motility	—	—	+	+	—	—
Salicin	+	+	+	+	—	—
Maltose	—	+	+	+	+	+
Trehalose	+	+	+	—	—	—
Lactose	—	—	—	—	—	+
Hippurate	—	+	—	—	—	—
Arginine	⊗	—	—	—	—	—

* ⊗, weak positive reaction; nt, not tested.

No strain forms gas in glucose media or grows at 45°. All strains grow at 15°. Colonies are rough (R) or intermediate or irregular (I).

All strains ferment glucose, fructose, mannose, cellobiose and galactose. No strain ferments sucrose, arabinose, xylose, melibiose, sorbose, raffinose, melezitose, rhamnose, mannitol, sorbitol, inositol, dulcitol, inulin, α -methyl mannoside or α -methyl glucoside. Aesculin is not hydrolyzed.

Group 3. The organisms contained in this group (Table 3) had a minimum temperature for growth at about 22° and a maximum at about 46° (except strain 33) and thus belonged to the high temperature homofermentative group, *Thermobacterium* Orla-Jensen (Orla-Jensen, 1919, 1943). The subsurface colonies in tomato agar were 'R' or 'I' in type but a few strains (particularly nos. 34–36) occasionally produced the variant smooth type of colony referred to above. Strains 24–33 can readily be identified with *L. acidophilus* (Curran, Rogers & Whittier, 1933; Rogosa *et al.* 1953) and its synonym *Thermobacterium intestinale* (Orla-Jensen, Orla-Jensen & Winther, 1936; Orla-Jensen, 1943), although strains 28–32 were unusual in being unable to

Table 3. *The characteristics of 13 strains of lactobacilli of group 3*

Test	Behaviour* of strain no.												
	24	25	26	27	28	29	30	31	32	33	34	35	36
Growth at 45°	+	+	+	+	+	+	+	+	+	—	+	+	+
Acid in milk (%)	0.6	0.5	0.5	0.3	<0.1	<0.1	<0.1	0.1	nt	0.2	0.4	<0.1	0.3
Form of lactic acid	DL	nt	DL	nt	DL	nt	DL	DL	nt	DL	nt	L(+)	nt
Mannitol	—	—	—	—	—	—	—	—	—	—	+	+	+
Raffinose	—	—	—	—	—	—	—	—	—	—	+	+	+
Melibiose	—	—	—	—	—	—	—	—	—	—	+	+	+
Trehalose	+	+	—	—	+	+	—	+	—	+	+	+	+
Galactose	+	+	+	⊗†	+	⊗†	⊗†	⊗	—	+	+	+	+
Lactose	+	+	+	+	—	—	—	+	—	+	+	+	+
Salicin	+	+	+	—	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	—
Aesculin	+	+	+	⊗	+	+	⊗	+	+	—	—	—	—

* ⊗, weak positive reaction; †, late fermentation; nt, not tested.

No strain forms gas in glucose media or grows at 15°. Colonies are rough (R) or intermediate or irregular (I).

All strains ferment glucose, fructose, mannose, sucrose and maltose. Strain 24 ferments α -methyl glucoside slowly. No strain ferments arabinose, xylose, sorbose, melezitose, rhamnose, inulin, inositol, sorbitol, dulcitol or α -methyl mannoside. Hippurate is not hydrolyzed and NH_3 is not produced from arginine.

ferment lactose. Representative cultures of both the lactose-fermenting and the lactose-negative types produced only DL-lactic acid.

Strains 34-36 differed from the others of group 3 in fermenting mannitol to an appreciable extent; they also fermented raffinose and melibiose rapidly and extensively. One strain tested produced L(+)-lactic acid. The fermentation of mannitol in the high temperature group of homofermentative lactobacilli is unusual, but it has been reported for *L. salivarius* (Rogosa *et al.* 1953) and for a high temperature variant of *L. plantarum* isolated from the caecal contents of turkeys (Harrison & Hansen, 1950); it was also reported for one strain of *L. lactis* (syn. *Thermobacterium lactis*) by Orla-Jensen (1943).

Strains 34-36 seem to resemble *L. salivarius* most closely and in particular the salicin-fermenting variety of that species. However, from the available descriptions it is difficult without the use of nutritional tests to differentiate this species from the high temperature variant of *L. plantarum* referred to above.

One further homofermentative strain had characters which were oddly individual. It was of the low temperature type, the only substrates attacked to an appreciable extent were glucose, fructose, mannose, salicin, cellobiose, trehalose, sucrose, maltose and melezitose, and the lowest pH value recorded in any fermentation test was 4.9, an unusually high figure for a homofermentative strain. Ammonia was produced from arginine.

Heterofermentative strains

Group 4. The majority of the heterofermentative isolates were placed here. Growth of the 13 group 4 strains occurred at 45° but not at 15° and with the exception of two strains the colony type was 'R' or 'I'. Ammonia was produced from arginine and less than 0.2% of acid was formed in milk. The following substrates were fermented: glucose, galactose, sucrose, lactose, maltose, melibiose and raffinose by all strains, arabinose by 12 strains, α -methyl glucoside by 10 strains, mannose (weakly) by 2 strains, xylose by 2 strains and fructose by 1 strain. The fermentation of α -methyl glucoside was in all cases delayed; a further 3 strains showed a questionable fermentation of xylose. Salicin, cellobiose, trehalose, sorbose, melezitose, rhamnose, inulin, mannitol, sorbitol, inositol, dulcitol and α -methyl mannoside were not attacked. Aesculin and hippurate were not hydrolyzed. The maximum temperature for growth was at about 46° and the minimum for most strains at about 24°; one strain was capable of growth at 20°.

The organisms of this group correspond most closely with *L. fermenti* (Pederson, 1938; Rogosa *et al.* 1953), although the fermentation of arabinose by a majority of strains is unusual. In many cases, however, the final pH was higher with arabinose than with glucose. A further unusual feature was the almost uniform inability of these strains to attack fructose, a sugar frequently fermented more readily than glucose. However such variations from the more usual behaviour of *L. fermenti* probably have little significance.

One strain of this group was unique in forming pigmented subsurface colonies. The pigment, which was deep orange-red in colour, was not water soluble.

Group 5. The 6 strains of this group differed from the preceding group in the following respects: growth occurred at 15° but not at 10°, colonies were of the 'S'

type, and all strains fermented mannose and fructose. Arabinose was fermented by 4 strains but none attacked xylose.

Although the temperature range of these isolates is that of *L. buchneri* (Pederson, 1938) certain of the characters are not typical of that species. Rogosa and his associates concluded from a study of a large number of strains, including some originally investigated by Pederson, that the fermentation of melezitose was a characteristic feature of *L. buchneri* (Rogosa *et al.* 1953): but no strain in group 5 attacked this substrate. Further, the action on pentoses does not seem completely typical of *L. buchneri* as defined by Pederson (1938), and the characters of these strains thus indicate that they occupy a position intermediate between *L. buchneri* and *L. fermenti*.

Unclassified strains. Five strains differed from the heterofermentative isolates already described in growing at 15° but not at 45° and in lacking the ability to produce ammonia from arginine. Aesculin and hippurate were not hydrolyzed. Three types were recognized. Type 1 (2 strains) had 'R' or 'I' colonies and the only substrates fermented to an appreciable extent were glucose, xylose and arabinose. Microscopically small irregular coccoid elements, similar to forms produced by propionibacteria under certain conditions of growth, were seen. However, lactate was not fermented and acetic acid was the only volatile fatty acid produced in their fermentation of glucose. It is possible that these organisms are more closely related to *Leuconostoc* than to *Lactobacillus*. Type 2 (2 strains) had 'S' colonies and both strains fermented glucose, fructose, trehalose, maltose and mannose. Microscopically the organisms were small, slender rods occurring in pairs; chains were infrequent. Type 3 (1 strain) had the 'S' type of colony. Glucose, fructose, galactose, trehalose and maltose were fermented to an appreciable extent and arabinose and xylose to a limited extent. The morphology was similar to that of type 2.

The dissimilation of malic and citric acids

Of the organic acids occurring in appreciable amounts in grass (perennial rye-grass) only malate and citrate are known to disappear during its ensilage (Hirst & Ramstad, 1957). It was thus of interest to determine the ability of silage strains of lactobacilli to attack these acids.

Citrate. A test for CO₂ production was used to detect citrate dissimilation; the agar closure technique described by Gibson & Abd-el-Malek (1945) was employed. Although attempts to use this method were not entirely successful, the following medium of several tried yielded the most promising results: peptone, 1 g; meat extract, 1 g; yeast autolysate, 5 ml; tomato extract, 10 ml; Tween 80, 0.05 ml; NaH₂PO₄, 0.1 g; salts mixture (Rogosa *et al.* 1951), 5 ml; agar, 0.2 g; citrate buffer, 10 ml; distilled water to 100 ml; pH 5.4. The citrate buffer was prepared by dissolving 2.5 g of citric acid in a little distilled water, adjusting the pH of the solution to *c.* 5.4 with 10 N KOH and making the volume up to 10 ml with distilled water. The inoculum used was 3–4 drops by capillary pipette and incubation was at 30° for 14 days. Occasional false negative results were obtained, apparently due to retention of CO₂ in the medium. That CO₂ had indeed been produced in such cases could be

shown by partly immersing the tubes in water at *c.* 85° when the agar seals were forced upwards by the released gas; this gas was absorbable by alkali.

Despite the limitations of the method, it was clear that all strains of *L. casei* produced CO₂ from citrate, a feature noted for this species by Gibson & Abd-el-Malek (1945) and by de Man (1956). However, in contrast with the reports of these workers, it was noted that several strains of *L. plantarum* (nos. 7-10, 14 and 15) also produced gas from citrate; a further three strains (nos. 4-6) appeared to liberate small amounts of CO₂. Other cultures yielding positive results in this test were two strains of group 2 (nos. 18 and 23) and most heterofermentative isolates. In the latter case 0.2% (w/v) of glucose was incorporated in the medium; a similar medium with no citrate added served as control.

Malate. Most previous workers who have tested for this feature have detected malate dissimilation either by analysis of the products formed or by noting the reduction of acidity which results from its breakdown. Preliminary experiments suggested that if a suitable medium were used a test for CO₂ production by the agar closure technique might be more satisfactory than either of the above procedures, especially when large numbers of cultures were being examined. Such a medium has been devised, and repeated testing over a period of three years has shown that the results obtained for homofermentative isolates are completely reproducible, though the method is less satisfactory for heterofermentative strains. The medium contains peptone, 1 g; meat extract, 1 g; yeast autolysate, 5 ml; tomato extract, 10 ml; Tween 80, 0.05 ml; glucose, 1 g; MnSO₄·4H₂O, 0.05 g; agar, 0.2 g; malate buffer, 10 ml; distilled water to 100 ml; pH 5.4. The malate buffer is prepared by dissolving 4 g of DL-malic acid in a little distilled water, adjusting the pH of the solution to *c.* 5.4 with 10 N KOH and making the volume up to 10 ml with distilled water. The final concentration of malate in the medium is thus about 0.3 M. For heterofermentative strains the glucose concentration is reduced to 0.2% (w/v) and a control medium lacking malate is also used. Malate in 0.3 M concentration is rather toxic at pH 5.4 for some of the high temperature homofermentative lactobacilli (incubated at 37°); for this group a concentration of 0.1 M *laevo*-malate has given good results. The lower concentration of malate occasionally gives false negative results with some strains of the low temperature homofermentative lactobacilli (incubated at 30°).

The inoculum used was 3-4 drops by capillary pipette of young cultures in BTB medium. In positive cultures the agar seal is completely separated from the remaining medium and is frequently blown up to the cotton plug.

Using the media and method described, all the strains of groups 1 and 2 and a majority of the heterofermentative isolates were found to liberate CO₂ from malate. With the group 3 organisms an interesting differentiation was obtained. Although all three strains (nos. 34-36) of the mannitol-fermenting type (? *L. salivarius*) produced gas from malate, no strain of *L. acidophilus* did so.

A few cultures of lactobacilli from sources other than silage have also been tested for their ability to produce CO₂ from malate. Positive results were obtained for three strains of *L. salivarius* (two strains kindly supplied by Dr. M. Rogosa and one, ATCC 11742, by Dr. M. E. Sharpe), one strain of *L. plantarum* var. *mobilis*

(kindly supplied by Dr. M. E. Sharpe), four strains of *L. casei* (including three strains of the high temperature variety), and *L. arabinosus* strain 17-5. The following cultures yielded negative results: one strain of *L. acidophilus*, *L. leichmannii* ATCC 4797, two strains from cheese tentatively identified as *L. lactis* and one strain from yoghurt tentatively identified as *L. helveticus* (or *Thermobacterium jugurt*).

It should be emphasized that in order to demonstrate CO₂ production from malate by the method described it is essential to use a medium containing a relatively high concentration of potassium. This requirement is conveniently satisfied by adding malate in the form of a potassium malate-malic acid buffer. In addition, some strains of *L. casei* and of *L. plantarum* did not show gas production by the agar seal method in the absence of added Mn⁺⁺.

DISCUSSION

The freshly harvested grass used for ensilage rarely had more than a thin seeding of lactobacilli. Only two of the six strains isolated from such material were homofermentative and both were found to be atypical strains of *L. plantarum* (nos. 13 and 15, Table 1). Strain 13 was representative of isolates from a herbage sample carrying an unusually high number of lactobacilli (acetate agar count, 5×10^3 /g dry wt of grass). The remaining grass isolates comprised one strain of *L. fermenti* (group 4, chromogenic strain), one strain of group 5 and two unclassified strains of types 1 and 2 respectively. Typical strains of *L. plantarum* were never encountered on herbage samples (quantities examined = 1/6 g of grass). That such organisms are of relatively infrequent occurrence on forage is further indicated by their low incidence in the laboratory silage. This finding is in essential agreement with that of Kroulik, Burkey & Wiseman (1955). Previous reports of the dominance of this species in silage and its relatively infrequent appearance in the laboratory silage examined in this work lend support to the view that chance contamination plays a large part in determining its occurrence on forage. It should be emphasized that during sampling and subsequent handling of herbage maximum care was taken to avoid its contamination. That the grass used for ensilage could support rapid and extensive growth of *L. plantarum* and of other silage isolates including *L. casei* was repeatedly demonstrated in laboratory inoculation experiments.

A further feature of this work which is at variance with most previous reports was the finding that heterofermentative strains formed a notable proportion of the lactobacilli encountered during the early stages of ensilage. In several batches of silage such types accounted for nearly half the total number of isolates. *L. brevis* was never isolated during the course of this work. In screening tests of a large number of isolates from laboratory silage, representative strains of the few low temperature heterofermentative types occurring were always retained for detailed study. These strains did not form a homogeneous group and none had the characters of previously described species.

Although only six strains of group 2 type were examined in detail it is possible that at least on some occasions they occur more frequently than is indicated by their proportionate representation in this study. In two batches of silage (incubated at 30°)

where screening tests were sufficiently detailed to permit recognition of members of this group, they constituted at least a major proportion of the lactobacillus count during the early days of ensilage.

The occurrence of *L. acidophilus* in a majority of the laboratory silages indicates that this species is not limited to a parasitic habitat as suggested by Davis (1956). That it can proliferate in silage has been clearly shown by the results obtained in this work.

The ability to dissimilate malate appears to occur more commonly in the lactobacilli than is indicated in the literature. The results secured here suggest that it is a usual feature of the low temperature homofermentative group but that it is probably uncommon in the high temperature homofermentative group with *L. salivarius* a notable exception. For the successful use of the cultural test described for the detection of CO₂ production from malate, the provision of Mn⁺⁺ and in particular K⁺ in amounts in excess of those normally required for growth is essential. Such mineral requirements are in accord with those described for the activity of the malic enzyme of *L. arabinosus* strain 17-5 (Nossal, 1951). The ability of many silage strains of lactobacilli to dissimilate malate in pure culture indicates that they may also do so in appropriate conditions in silage. The data of Macpherson, Wylam & Ramstad (1957) suggest that on at least one occasion lactobacilli appeared to play some part in the disappearance of malate in ensiled grass.

A notable feature of the isolates described is the relatively high proportion of unusual types, particularly in the low temperature homofermentative group. This finding cannot be ascribed to the methods of study used. About half the strains attached to *L. plantarum* were atypical, mainly in lacking the ability to ferment maltose and mannitol. Although further variations occurred, these were in characters usually recorded as variable for this species.

It has been suggested by Rogosa *et al.* (1953) that *L. arabinosus* (Fred, Peterson & Anderson, 1921b) be regarded as a distinct species. *L. arabinosus* has usually been considered a synonym of *L. plantarum* (Pederson, 1936). Although the characters of one strain (no. 8, Table 1) of the present study agreed closely with the extended definition of *L. arabinosus* (Rogosa *et al.* 1953) further strains differed in one or more respects, making a distinction between the *L. arabinosus* type and other strains of *L. plantarum* somewhat obscure. These data suggest that a separation of the two types as distinct species would not be desirable at the present time. De Man (1956) has reached a similar conclusion.

A comparison of the characteristics of silage strains with the species definitions of heterofermentative lactobacilli given by several authors indicates that new isolates cannot be identified with any degree of precision. In particular the use of tests for growth at 15° (or 16°) and 45° as a means of defining the temperature requirements of such organisms appears to have a limited value in differentiation. For example, the reports of different investigators suggest that *L. brevis* may grow at 15° only, or at 45° only, or at both of these temperatures to some extent. The *Betabacterium breve* of Orla-Jensen, however, had a temperature range of c. 15°–38° (Orla-Jensen, 1919). Similar confusion exists in the literature with regard to the temperature requirements of *L. buchneri*. It would thus appear that a more detailed examination

of the limiting temperatures for growth of heterofermentative types would yield information of a more useful character than that provided by tests for growth at 15° and 45° only.

A majority of the heterofermentative strains isolated from laboratory silage formed two more or less compact groups. The major difference between the two groups was in the lower limiting temperature for growth: but in view of the uncertainty attached to the value of temperature relationships in differentiating the heterofermentative lactobacilli it is difficult to assess the significance of this difference.

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The Editors of The Journal of Applied Bacteriology do not accept responsibility for the Reports of the Society's Proceedings. Abstracts of papers read are published without verification of the scientific content. As a rule where no abstract follows the title the paper read has formed the basis of a full length paper in the Journal.

THE SOCIETY FOR APPLIED BACTERIOLOGY

WINTER MEETING

The Winter Meeting was held in the Barnes Hall of The Royal Society of Medicine, 1, Wimpole Street, London, W.1, on Wednesday, 14 January, 1959. In the morning there was a short business meeting, followed by a paper reading session. In the afternoon there was a Joint Meeting with the Microbiology Group of the Society of Chemical Industry, when the subject under discussion was 'Differential Media'.

Morning Session. The following papers were read:—

The Isolation of Virulent Staphylococci from Weekly Composite Milk Samples. By A. H. WALTERS (*Deosan Ltd., Research Laboratory, 42, Weymouth Street, London, W.1*). (See *J. appl. Bact.* **22**, 248).

The Multiplication of Coli-aerogenes Organisms in Milk held at Refrigerator Temperatures. By J. J. PANES and S. B. THOMAS (*National Agricultural Advisory Service, Trawscoed, Aberystwyth*). (See *J. appl. Bact.* **22**, 272).

A Test to Differentiate *Pseudomonas* from Other Gram-Negative Bacteria. By MARGARET J. THORNLEY (*Low Temperature Research Station, Downing Street, Cambridge*).

Some 400 strains of Gram-negative bacteria isolated from chicken meat stored at low temperatures were identified by existing methods. Of these, 154 were considered to be *Pseudomonas*, 188 *Achromobacter* and 44 others belonged to the coli-aerogenes group or remained unclassified. A new test for the differentiation of *Pseudomonas* from *Achromobacter* and related genera has been developed, consisting of growth under a vaseline seal in a semisolid medium containing arginine. A positive reaction is shown by a rise in pH, considered to be due to the action of arginine dihydrolase. Of the 154 *Pseudomonas* strains, 153 gave positive results in this test, while 1 *Pseudomonas* and 188 *Achromobacter* strains were negative. When named strains from culture collections were tested, 49 *Pseudomonas* strains were positive, while 2 plant pathogenic pseudomonads were negative. All *Achromobacters* tested were negative as were 4 *Alcaligenes* species. Other organisms which gave positive results could be distinguished from *Pseudomonas* by their action in glucose, *Alcaligenes bookeri* being inactive, whereas *Aeromonas* and *Vibrio* were fermentative.

Studies on Aerosol Disinfection of Poultry Premises. By D. A. MCKENZIE, JEAN LAMBERT and J. GETTY (*The North of Scotland College of Agriculture, 41½, Union Street, Aberdeen*). (See *J. appl. Bact.* **22**, 258).

The Growth of *Penicillium chrysogenum* in Continuous Culture. By S. J. PIRT and D. S. CALLOW (*Microbiological Research Establishment, Experimental Station, Porton, Salisbury, Wilts*).

A filamentous mould was cultured by the continuous flow method in which medium is supplied at a constant rate and the culture volume is kept constant. Flow rates up to 0.1 culture volumes/hr were used. The mycelial dry weight concentration and the yield of mycelium/g of carbon source were equal to or slightly greater than the maximum obtained in batch culture. With glucose concentrations up to 80 g/l. at a flow rate of 0.05 culture volumes/hr, about 45% of the substrate carbon was converted into mycelial carbon and the remainder oxidized to CO₂.

With unlimited amounts of all nutrients available growth of the mould followed the exponential law, like growth of bacteria, and therefore the mould had a constant doubling time. The minimum doubling time was 4.9 hr.

The oxygen demand of the mould as a function of growth rate was determined.

Conditions were found under which the rate of penicillin production/g of mycelium remained at its maximum value for 1000 hr.

A Two-Stage Continuous Culture Apparatus. By D. S. CALLOW and S. J. PIRT (*Microbiological Research Establishment, Experimental Station, Porton, Salisbury, Wilts*).

The two-stage continuous flow apparatus described has a first stage working volume of 2 to 3 l. and a second stage working volume of 6 to 14 l. Each stage has separate controls over the environmental factors, temperature, pH value and nutrient supply, and over foaming. In the first stage the dilution rate is altered by changing the flow rate: in the second stage the dilution rate is altered by changing the working volume.

The apparatus has proved suitable for the continuous culture of a filamentous mould, which is more exacting from the engineering standpoint than culture of bacteria.

Studies Arising from the Observation of Osmophilic Yeasts by Phase Contrast Microscopy. By M. PAMELA SCARR and ELISABETH COSTER (*Tate & Lyle, Ltd., Research Laboratories, Keston, Kent*).

As cell density varies with age in osmophilic yeasts growing in concentrated sugar solutions, Barer & Joseph's phase contrast technique has been shown to estimate cell activity of osmophils in such solutions, as such or spread over inert bodies to simulate raw sugar. All the osmophilic yeasts examined reacted to this technique but the concentration at which there is the closest relationship between per cent active cells determined by phase contrast and by standard methods varies from 52–65°Bx with the species used.

The technique applied to syrups containing *Saccharomyces rouxii* showed a limited growth. When the syrup is present as a film the growth can spread outwards, as has been observed in raw sugar not stored under controlled R.H.

Afternoon Session. The following papers were read at the Joint Meeting with the Microbiology Group of the Society of Chemical Industry on the subject of

Differential Media

The Use of Antibiotics in Media for Assessing Bacterial Infection in Food Yeast. By M. FLEMING, N. H. BARNARD and L. A. ALLEN (*Tate & Lyle, Ltd., Research Laboratories, Keston, Kent*).

Selective Media Used in the Microbiological Examination of Sugar Products. By M. PAMELA SCARR (*Tate & Lyle, Ltd., Research Laboratories, Keston, Kent*).

Selective Media for Yeasts and Bacteria. By F. W. BEECH and J. G. CARR (*Research Station, Long Ashton, Bristol*).

The Enumeration and Identification of Some Types of Anaerobic Bacteria Occurring in Foods. By D. A. A. MOSSEL (*Central Institute for Nutrition and Food Research T.N.O., Utrecht, The Netherlands*).

Media for Lactobacilli. By M. ELISABETH SHARPE (*National Institute for Research in Dairying, Shinfield, Reading, Berks*).

Differential and Selective Media for the Faecal Streptococci. By ELLA M. BARNES (*Low Temperature Research Station, Downing Street, Cambridge*).

Differential Media for Sulphur Bacteria. By J. POSTGATE (*National Chemical Laboratory, Teddington, Middlesex*).

Culture Media for Fungi. By G. SMITH (*Biochemistry Department, London School of Hygiene and Tropical Medicine, Gower Street, London, W.1*).

ANNUAL GENERAL MEETING AND CONFERENCE

The 29th Annual General Meeting and Conference was held in Bedford College (University of London) on 7, 8 and 9 July, 1959. Members assembled on Monday, 6 July, and the following day was devoted to a Symposium on 'The Lactobacilli', to which two visitors from abroad contributed. On Wednesday, 8 July, the Annual General Meeting was held, followed by a paper reading session which occupied the rest of the morning. In the afternoon members visited either the Cadby Hall works of J. Lyons & Co., Ltd. or the Laboratories and Works of T. Wall & Sons, Ltd., and in the evening there was the Annual Society Dinner. Thursday, 9 July, was occupied by further paper reading sessions.

The following papers were read on 7 July in the Symposium on

The Lactobacilli

An Approach to the Classification of the Lactobacilli. By M. ROGOSA and M. ELISABETH SHARPE (*National Institute of Dental Research, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland, U.S.A. and National Institute for Research in Dairying, Shinfield, Reading, Berks*). (See *J. appl. Bact.* **22**, 329).

The Differentiation of Some Species and Strains of Lactobacilli by Chromatography. By G. C. CHEESEMAN (*National Institute for Research in Dairying, Shinfield, Reading, Berks*). (See *J. appl. Bact.* **22**, 341).

Lactobacilli of the Human Mouth. By G. H. G. DAVIS (*Bacteriology Department, The Medical School, The University, Birmingham*). (See *J. appl. Bact.* **22**, 350).

Factors Affecting Lactobacilli and their Implantation in the Intestine. By H. B. HAWLEY, P. A. SHEPHERD and DOROTHY M. WHEATER (*Research Department, Aplin & Barrett, Ltd., Yeovil, Somerset*). (See *J. appl. Bact.* **22**, 360).

Some Micro-Organisms from Chicken Meat Showing Relationships to both Lactobacilli and to Aerobic Sporeformers. By MARGARET J. THORNLEY and M. ELISABETH SHARPE (*Low Temperature Research Station, Downing Street, Cambridge and National Institute for Research in Dairying, Shinfield, Reading, Berks*). (See *J. appl. Bact.* **22**, 368).

The Anomalous Behaviour of Some Cider Lactobacilli. By J. G. CARR (*Research Station, Long Ashton, Bristol*). (See *J. appl. Bact.* **22**, 377).

Some Studies on Lactobacilli Causing Malolactic Fermentation in Wines. By H. LÜTHI (*Wädenswil, Switzerland*). (See *J. appl. Bact.* **22**, 384).

Studies on Beer-Spoilage Lactobacilli. By D. H. WILLIAMSON (*The Brewing Industry Research Foundation, Nutfield, Redhill, Surrey*). (See *J. appl. Bact.* **22**, 392).

The Properties and Classification of Some Lactobacilli Isolated from Grass and Silage. By R. M. KEDDIE (*School of Agriculture, Edinburgh*). (See *J. appl. Bact.* **22**, 403).

The following papers were read on 8 July:—

The Significance in Food Hygiene of *Lactobacillus bifidus*. By D. A. A. MOSSEL (*Central Institute for Nutrition and Food Research T.N.O., Utrecht, The Netherlands*). (See *J. appl. Bact.* **22**, 184).

Microbiological Aspects of the Genus *Microbacterium*. By PATRICIA A. TURBUTT, A. SEAMAN and M. WOODBINE (*Microbiological Unit, School of Agriculture, Sutton Bonington, Loughborough*).

The genus *Microbacterium*, in which perhaps the commonest non-sporing thermophilic bacteria are represented, contains the species *M. lacticum*, *M. flavum* and *M. liquefaciens*. On the basis of fermentation reactions, gelatin liquefaction and antibiotic susceptibility, 21 strains were divided into 18 *M. lacticum* and 3 *M. liquefaciens* (these being clearly the most resistant to penicillin and bacitracin).

The thermal resistance of these strains, using broth and milk media, indicated that *M. liquefaciens* had a lower resistance (under 65°) than *M. lacticum*. It is suggested that the recommended temperature (75°) for separating *Microbacterium* spp. may be within a critical zone for *M. lacticum* and that 68° for 30 min would be better, as it is above the critical zone for *M. liquefaciens* (63–5°) and below that for *M. lacticum* (70–83°).

The Early Development of Dairy Microbiology. By A. SEAMAN (*Microbiological Unit, School of Agriculture, Sutton Bonington, Loughborough*).

The Microbiological Assay of Thiamin—A Modified Method. By PAT M. SCHOLDS (*Bacteriology Department, Queen Elizabeth College, Campden Hill Road, London, W.8*).

A microbiological assay of thiamine was described in which 2–8 µg thiamine/tube can be estimated.

The method employed is a modification of that of Fitzgerald & Hughes (1949) using *Lactobacillus fermenti* 36, or, alternatively, a modification of the *L. viridescens* assay of Deibel, Evans & Niven (1957).

The observation (Deibel *et al.*) that *L. viridescens* is less sensitive than *L. fermenti* 36 to inhibitory and stimulatory substances other than thiamine was confirmed. The assay with *L. viridescens* may therefore be preferred.

The modified assay is carried out in 2 ml amounts in 8×80 mm EEL colorimeter tubes and is useful when only small quantities of test material are available.

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A Note on the Maintenance of Assay Organisms as Freeze-Dried Cultures.
 By PAT M. SCHOLDS and W. S. GREAVES (*Bacteriology Department, Queen Elizabeth College, Campden Hill Road, London, W.8 and National Chemical Laboratory, Teddington, Middlesex*).

Preliminary experiments have indicated that it may be possible to maintain microbiological assay organisms as freeze-dried cultures and to use such cultures as direct inocula for assays.

The organisms employed in this work were: *Lactobacillus arabinosus* 17/5 for nicotinic acid assay and pantothenic acid assay; *Pediococcus cerevisiae* 8081 for folinic acid assay; *L. fermenti* 36 for thiamine assay; *L. viridescens* for thiamine assay; *Streptococcus faecalis* for folic acid assay and *L. helveticus* for riboflavin assay.

The suspending medium for freeze-drying was in each case 3 parts of horse serum plus 1 part of 30% glucose broth. Assays were carried out up to 3 months after freeze-drying to compare the results obtained with three types of inoculum: (i) freeze-dried culture washed and resuspended directly in saline, (ii) freeze-dried culture subcultured once and (iii) standard inoculum subcultured from a stock agar stab. In general inoculum (ii) gave poor results; but where freeze-dried culture was used as direct inoculum the results were very similar to those obtained with inoculum derived from the stock agar stab. The results of the first three assays listed above were particularly promising.

The following papers were read on 9 July:—

Benzoate-Resistant Yeasts. By M. INGRAM (*Low Temperature Research Station, Downing Street, Cambridge*).

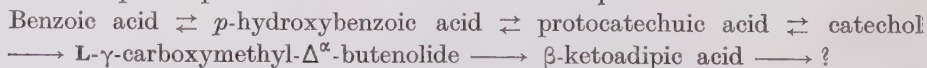
From three separate outbreaks of fermentation in commercial citrus squashes preserved with 500 p/m of benzoic acid, yeasts resembling a type strain of *Saccharomyces acidifaciens* were isolated. The three strains differed in minor respects; but all of them, and the type strain (though more slowly), were shown to grow in the presence of 500 mg/l. of benzoic acid at pH 3.5 in synthetic media, especially with 300 g/l. of sugar.

The yeasts were virtually incapable of respiring or assimilating benzoic acid alone, but could apparently do so in the presence of sugars.

As a result of their growth, the concentration of benzoic acid fell from 500 to 350–400 mg/l., and the following could be demonstrated:—

- (i) Substances chromatographically similar to *p*-hydroxybenzoic acid (A), protocatechuic acid (B) and catechol (C) were produced.
- (ii) From A, B and C were produced; from B, A and C; and from C, B and A.
- (iii) With benzoic acid a strong absorption developed at 230 mμ, the wavelength corresponding to L-γ-carboxymethyl-Δ^α-butenolide, a possible intermediate; and
- (iv) a Rothera reaction developed, the substance responsible behaving chromatographically like β-ketoadipic acid.

There is thus presumptive evidence for the reaction sequence:—



Bacteria of the *Aeromonas-Kluyvera* Group. By B. P. EDDY (*Low Temperature Research Station, Downing Street, Cambridge*).

These broadly resemble coli-aerogenes bacteria in producing gas from many carbohydrates anaerobically, but they have polar flagella. *Kluyvera* is defined as methyl-red

positive; not producing acetylmethylcarbinol, i.e. Voges-Proskauer negative. *Aeromonas* is defined as methyl-red negative; producing 2:3-butanediol (and presumably also acetylmethylcarbinol and thus V.-P. positive).

About 10 strains from various sources have been subjected to a preliminary examination. Most do not fit precisely into either of the two generic definitions, which seem inadequate to cover the group as a whole.

Almost all the strains grow on the common media selective for the coli-aerogenes group, more at 30° than 37°, and those which ferment lactose give presumptively positive reactions. This could be confusing, because attempts to isolate these organisms from mammalian faeces have so far failed. None however have resembled *Escherichia coli* type I.

It is suggested that these organisms may be not uncommon, but are usually mistaken for more or less aberrant coli-aerogenes bacteria. Features which help to distinguish them from coli-aerogenes bacteria, some of which are possessed by all the organisms yet examined, are digestion of soluble starch, peptonization of milk, rapid liquefaction of gelatin and positive oxidase (Kovacz) reaction, besides the polar flagellation.

Enterococci in Calves' Faeces. By A. WILSSENS and A. DE VLEESCHAUWER.
(*Rijkslandbouwhogeschool, Leerstoel voor Technologie, Gent, Belgium*).

A qualitative study has been made of the enterococcal flora of calves' faeces. The age of the calves varied between a few days and three months. The diet given was as follows: at first they were fed on colostrum and whole milk, but later a replacer was substituted gradually for the milk. The faecal samples were collected by means of rectal swabs. Isolations were made on the medium of Barnes, and the Hajna & Perry and Rosenow media were used for enrichment cultures.

The various strains isolated could then be divided according to their biochemical properties. We distinguished the following quantitatively important types: the established species *Streptococcus faecalis* and its variant *Str. faecalis* var. *liquefaciens*, the so-called *Str. faecium*, an aberrant sorbitol positive type of *Str. faecium* and the so-called unclassified strain no. 1 of Barnes & Ingram. We did not look for *Str. bovis*.

A total of 40 samples was examined. *Str. faecalis* was found in all of them, *Str. faecalis* var. *liquefaciens* in 2, *Str. faecium* in 22, aberrant *Str. faecium* strains in 10 and unclassified no. 1 in 12.

Changing the diet from milk produced changes in the type distribution. There was a tendency for *Str. faecalis* to disappear from the stools. In our opinion the best differential tests for the enterococci were: sensitivity to 1:2500 potassium tellurite, the fermentation of arabinose, TTC reduction (Barnes) and the fermentation of sorbitol.

This study has indicated that in very young calves *Str. faecium* is the predominant organism, in contrast to the enterococcus flora of bovine faeces where *Str. faecalis* is practically absent, and that more information is needed on the influence of various diets on the intestinal flora.

The Behaviour of Individual Bacteria in the Lag Phase. By L. B. QUESNEL and G. C. WARE (*Department of Bacteriology, University of Bristol, Canynge Hall, Whatley Road, Bristol, 8*).

A 'closed' (Herbert, 1960) continuous culture system (modified after Powell, 1955) was used to observe by phase contrast microscopy the generation times (within ± 2 min) of individual *Escherichia coli* growing in synthetic medium on a cellophane membrane. The inoculum was prepared, and all subsequent manipulations and observations performed, at 37°.

Generation times varied greatly and histograms revealed inter-quartile distances of 21 min for the second generation, reducing to 17 in the third and 13 in the fourth generation. Mean generation times for these generations were 56, 55, and 49 min respectively. The time from the moment of inoculation to the first division of each individual (1st-division time) was usually considerably longer than the mean generation time. Of the organisms in the inoculum 10% were non-viable, but of cells produced subsequently only 3% were non-viable.

We consider initial non-viability to be a result of shock on transfer to new medium; long 1st-division times and lengthened 2nd, 3rd, and 4th generation times contribute to the lag phase of bacterial growth.

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Some Difficulties in the Radioactive Labelling of Streptomycin. By G. C. WARE (*Bacteriology Department, University of Bristol, Canynge Hall, Whatley Road, Bristol, 8*).

AUTUMN MEETING, 1959

For the Autumn Meeting of the Society, held on Tuesday, 27 October, 1959, a visit was paid to the Central Veterinary Laboratory, New Haw, Weybridge, by kind permission of the Director.

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